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Prevention of Postoperative Adhesions in the Dog by Intravenous Injections of Plasminogen Activators.* (21921)

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Recent studies have indicated that experimental venous thrombi in the rabbit(1), and arterial thrombi in the dog(2) can be successfully liquefied *in vivo* by the intravenous administration of plasminogen activators.‡ Activation of animal plasminogen was carried out in the above studies by large amounts of streptokinase (SK). In addition, in the dog, plasminogen activation was carried out by injections of a mixture of small amounts of SK and a partially purified human plasminogen preparation(2). This latter method was based on the observation that the activation of animal plasminogen by SK was markedly facilitated by the presence of small amounts of human plasminogen preparations(5).§

The purpose of this report is to present evidence that, in dogs with an experimental traumatic peritonitis, intravenous injection of large amounts of streptokinase alone, or of a mixture of small amounts of SK and a human plasminogen preparation was capable of preventing or modifying the production of postoperative abdominal adhesions.

Materials and methods. Sixty-three mongrel dogs weighing from 10 to 15 kg were used in these experiments. A traumatic hemorrhagic fibrinous peritonitis was produced by scarifying a 2 x 2 inch area of peritoneal wall with a vegetable grater according to the technic of Wright and associates(6). Immediately following surgery, each animal was injected intramuscularly with 1,000,000 units of a long acting penicillin (Bicillin®). The animals were divided into 3 groups: 23 controls, 32 animals injected intravenously with SK‖ alone; and 8 animals injected intravenously with a mixture containing 25,000 units of SK and 60 mg of a partially purified human plasminogen preparation.¶ Of the animals receiv-

* Aided by grants from the National Institute of Arthritis and Metabolic Disease, National Institutes of Health, U. S. Public Health Service, and Lederle Laboratories Division, American Cyanamid Co.

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‡ Plasminogen refers to the naturally occurring precursor of plasmin, the proteolytic enzyme of mammalian plasma(3). Other terminology, in current use, includes profibrinolysin for the precursor and fibrinolysin for the active enzyme(4).

§ While 200 units of SK per ml of dog plasma was required to activate significant amounts of plasminogen, the addition of minute amounts of human plasminogen preparations to dog plasma reduced the SK requirement to approximately 20 units per ml of dog plasma(2).

‖ A commercial preparation (Varidase) Lot No. 7-1089-413, containing streptokinase and streptodornase, and obtained through the courtesy of Dr. J. M. Ruegsegger of Lederle Laboratories. This preparation also contains streptococcal hyaluronidase as well as other contaminating substances.

¶ A lyophilized human plasminogen preparation prepared from human plasma Fraction III (Cohn) by the Christensen and Smith technic(7). The preparation contained 4% nitrogen and had a proteolytic assay, following streptokinase activation, of 4.2 casein units per mg nitrogen(8).

TABLE I. Summary of Results of I.V. Plasminogen Activators on Prevention of Postoperative Adhesions in Dogs.

Animals	No. of dogs	Results*† No. with dense adhesions	No. with moderate adhesions	No. with no adhesions
Controls	23	19	4	0
SK alone—total group	32	10	5	17
400,000 u. od x 6	2	1	1	0
300,000 u. bid x 2	7	4	0	3
300,000 u. bid x 3	12	5	2	5
400,000 u. bid x 3	11	0	2	9
SK + human plasminogen prep.‡	8	0	6	2

* Classified according to thickness, firmness of adherence, and extent of adhesions as follows: Dense—thick, firmly adherent and covering entire area of abrasion. Moderate—thin, more loosely adherent, and scattered. None—occasional filmy to no adhesions.

† Dividing the entire data into 2 classes (*i.e.*, adhesions vs. no adhesions) and employing the chi square test (R. x 2 table; using Yates' correction) statistical analysis showed a highly significant difference between all treatment groups together as compared to the control animals (P less than .01).

Because it was believed that a trend was present relating SK dosage to suppression of adhesion formation, chi square testing of the controls against the combined 3 lower daily dose levels and against the highest daily dose level was carried out. Results indicated that the first 3 treatment groups combined, differed from the control in a manner to which little significance could be ascribed ($P = 0.05$). The last treatment group however showed a highly significant difference from the control (P less than .001).

The authors are indebted to Dr. Stanley Lang for the statistical treatment of the data.

‡ Injection mixture contained 25,000 u. SK and 60 mg of partially purified human plasminogen preparation.

ing SK alone, 2 were given a single injection of 400,000 units of SK daily for 6 days; 7 received 300,000 units twice daily for 2 days; 12 were injected with 300,000 units twice daily for 3 days; and 11 were given 400,000 units twice daily for 3 days. The animals receiving the activation mixture of SK plus the human plasminogen preparation were given single daily injections for 3 days. Treatment with the plasminogen activators was begun on the first postoperative day. Venous specimens of whole blood were obtained just prior to and 10 minutes following each injection of the plasminogen activator. The specimens were incubated at 37°C in a water bath and observed for clotting and subsequent lysis of the clot. The animals were sacrificed 12 days postoperatively and the extent and nature of the adhesions were noted. Specimens of the traumatized site, heart, lungs, kidneys, liver and spleen were taken for histological study.

Results. A summary of the results is shown in Table I. All of the 23 control animals developed adhesions, dense in 19, and moderate in 4. In the group of 32 animals receiving SK alone, 17, or approximately half, developed no adhesions, 5 had moderate, and 10 had dense adhesions. The prevention of adhesions was related to the daily dose of SK rather than the duration of treatment, with the best results occurring with the highest dose level of SK. Nine of 11 animals, receiving 400,000 units twice daily for 3 days, did not develop any adhesions. In the group of 8 animals receiving the mixture of 60 mg of the partially purified human plasminogen preparation and 25,000 units of SK, 2 did not develop adhesions, and the other 6 developed moderate adhesions. None of the animals of this latter group exhibited dense adhesions.

In order to define more clearly the effect of I.V. streptokinase on the peritoneal exudate, 3 control animals, and 3 animals receiving 400,000 units SK bid for 3 days, were sacrificed on the fifth postoperative day and the abraded sites examined histologically. Each of the control animals showed an extensive fibrinous exudate with evidence of rapidly developing organization. The exudate in each of the SK-treated animals was minimal with little evidence of organization. The striking

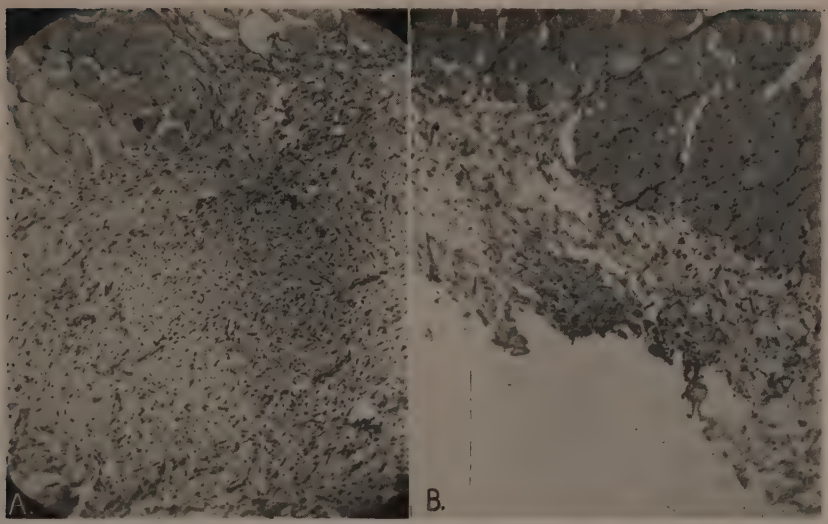

FIG. 1. Effect of I.V. SK on experimental traumatic peritonitis in the dog (see text). Specimens taken from abraded area on 5th postoperative day. A. Control animal. Note thick organizing granulation tissue adjacent to striated muscle fibers in cross section with penetration between muscle bundles (magnification 67 ×). B. SK treated animal. Thin layer of granulation tissue with little evidence of organization (same magnification).

histological difference in a control as compared to an SK-treated animal is shown in Fig. 1.

Each of the blood specimens removed prior to the injection of the activators clotted and did not significantly lyse over an observation period of 48 hours. Following the injection of SK alone, the blood clotted normally and subsequently lysed completely in from one to 4 hours.** Following injection of the mixture of SK and the human plasminogen preparation, the blood either did not clot, or the clot was poorly formed and rapidly lysed over the next 30 minutes. In several instances, determinations were made of plasma fibrinogen, plasminogen, and circulating proteolytic activity, with results similar to those previously reported with these 2 methods of plasminogen activation(2).

A finding of interest observed in the animals receiving SK alone related to the laparotomy wound. Preliminary studies revealed a high incidence of wound dehiscence in animals receiving large doses of SK alone (300,000 units bid). The dehiscence was attributed to an accelerated resorption of the catgut sutures. With the use of silk sutures, as utilized in the studies reported, no wound dehiscence was noted. However, in the animals treated with the largest daily dose of SK alone (400,000 units bid), fresh hemorrhage into the incisional wound was observed during the 3 days of SK therapy. Following the cessation of SK administration, the hemorrhage rapidly resorbed. At the time of final exploration, the wounds were well healed and did not differ from the control wounds.

No other significant toxic manifestations were noted in any of the animals. All the animals survived the total observation period. Studies of the sections of heart, lung, kidney and liver were not remarkable.†† In the majority of the animals, sections of the spleen revealed passive congestion but the presence of congestion was independent of treatment.

Discussion. The prevention of postopera-

** Lysis times of 1-2 hours were observed after the injection of 400,000 units of SK and 3-4 hours after the injection of 300.000 units of SK.

†† We are indebted to Dr. Philip Wasserman, Department of Clinical Laboratories, Jewish Hospital of Cincinnati, for these studies.

tive abdominal adhesions in many of the animals receiving the intravenous injection of plasminogen activators probably indicates a lysis of the traumatic inflammatory exudate. It is highly unlikely that the effects of the plasminogen activators can be entirely attributed to a lack of formation of a fibrinous exudate since therapy was not instituted for a full day following trauma. The presence of increased capillary permeability in the traumatic area may account for the appearance of plasmin or of SK at the site of exudation. Although the streptokinase preparation contained streptodornase and streptococcal hyaluronidase, both of which may contribute to the lysis and resorption of the exudate, we have stressed the fibrinolytic system since fibrin is the major insoluble component of the experimental exudate in this study.

The differences observed with the 2 types of activators used in this study, and the special fibrinolytic activity seen after *in vivo* injections of SK alone, have been previously noted (2). Recent studies indicate that the mixture of SK plus the human plasminogen preparation supplies the activator for the animal plasminogen(5,9). The special fibrinolytic effects seen with large doses of SK alone are related to the adsorption of SK on fibrin, and the ability of SK to suppress antiplasmin activity(10). The resultant of these latter phenomena is to provide an "inhibitor free" fibrin for the action of activated plasmin(10).

Conclusion. In dogs with an experimental traumatic peritonitis, the intravenous injection of streptokinase alone in large doses, or of a mixture of smaller amounts of SK plus a partially purified human plasminogen preparation, prevented or modified the development of postoperative adhesions.

1. Johnson, A. J., and Tillett, W. S., *J. Exp. Med*, 1952, v95, 449.

2. Sherry, S., Titchener, A., Gottesman, L., Wasserman, P., and Troll, W., *J. Clin. Invest.*, 1954, v33, 1303.

3. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, v28, 559.

4. Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, v12, 1.

5. Sherry, S., *J. Clin. Invest.*, 1954, v33, 1054.

6. Wright, L. T., Smith, D. H., Rothman, M., Quash, E. T., and Metzger, W. I.. PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 602.

7. Christensen, L. R., and Smith, D. H., Jr., *ibid.*, 1950, v74, 840.

8. Remmert, LeM., and Cohen, P. P., *J. Biol. Chem.*, 1949, v181, 431.

9. Troll, W., and Sherry, S., *ibid.*, 1955, v213, 881.

10. Sherry, S., in preparation.

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Electrophoretic Distribution of Hexosamine in Plasma Proteins of the Rat Following Thyroidectomy.* (21922)

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Plasma hexosamine levels increase in the rat following either thyroidectomy or hypophysectomy(1). The cause appears to be a deficiency of thyroid hormone in both instances. Hexosamine is present in all plasma protein fractions but is richest in the globulins(2). Since globulins have been reported to be elevated in rats following thyroidectomy (3), electrophoretic studies were carried out to establish which protein fractions were responsible for the plasma hexosamine changes observed in the rat following thyroidectomy.

Methods. Male Sprague-Dawley rats, weighing 55-65 g, were individually caged, fed a high calcium diet(4), and permitted to drink water *ad libitum*. Thyroidectomy was per-

* The author wishes to thank Mr Joseph B. Foley for his invaluable technical aid.

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TABLE I. Electrophoretic Distribution of Plasma Proteins and Hexosamine in Plasma Proteins of Normal and Thyroidectomized Rats.*

Group	Albumin	Globulins α-1	α-2	β	γ	Total
		Protein (% of total)				
Control	31.8 ± 0.2	18.1 ± .7	14.0 ± .3	17.5 ± .7	18.6 ± 1.3	—
Thyroidectomy	29.7 ± 1.0	16.3 ± .8	12.7 ± .6	18.8 ± 1.1	22.6 ± 1.0	—
Significance (P)	<.10	<.15	<.10	<.40	<.03	
		Hexosamine (mg/100 ml of plasma)				
Control	5.7 ± 0.8	48.2 ± 3.1	21.4 ± .7	19.6 ± 1.3	9.7 ± 1.4	104 ± 4.8
Thyroidectomy	8.8 ± 1.1	50.6 ± 2.4	21.3 ± .7	28.6 ± 2.1	18.8 ± 1.5	128 ± 4.6
Significance (P)	<.05	>.50	>.50	<.01	<.01	<.01
		Hexosamine (% of protein)				
Control	.30	4.42	2.55	1.87	0.87	1.73

* Mean ± stand. error. Each group contained 6 rats.

formed under ether anesthesia. All rats were weighed on the day of operation and at 12 and 35 days following the day of operation. The thyroidectomized rats used for study were selected on the basis of plateaued growth curves, a measure of complete removal of the thyroid gland. Blood samples were obtained 35 days after the day of operation from the external jugular vein under light ether anesthesia, using oxalate as an anticoagulant. Hematocrit and hexosamine determinations were carried out as previously described, with plasma hexosamine levels being corrected for hematocrit changes(4,5). Electrophoretic separation of the plasma proteins was carried out on filter paper in an apparatus similar to that used by Durrum(6). Whatman No. 1 paper sheets or strips with a resistance area of approximately 500 cm^2 were used. Electrophoretic separations were carried out at room temperature, at constant current (10mA) in pH 8.6 veronal buffer (ionic strength 0.06) for 11-16 hours. The voltage varied with conditions of evaporation in the system. For protein studies 0.01-0.02 ml of plasma were applied on a 25 mm line. For hexosamine studies 0.1 ml was applied to a 150 mm line. At the completion of a run the paper was dried in an oven at 110°C for 20 minutes, stained for 7 minutes in an alcoholic solution of 0.1% bromphenol blue saturated with mercuric chloride(6), rinsed in tap water and dried again in an oven at 110°C. For protein studies the strip was read at 600 mμ in a Beckman spectrophotometer (model DU) by means of a specially constructed strip holder. A protein distribution curve was obtained by charting optical density (ordinate) against distance migrated (abscissa). The protein distribution was measured by cutting out the charted electrophoretic curve, weighing it and relating the weight of each component to the total weight.‡ The electrophoretic distribution of hexosamines was determined by cutting the paper according to the known protein bands. Each band of paper was hydrolyzed in 3 ml of 1N hydrochloric acid for 15 hours and after isolation on Dowex-50, hexosamine was determined by a modification of the method of Elson and Morgan(5).

Results. The distribution of plasma proteins following thyroidectomy is shown in the upper part of Table I. There was a significant increase in the relative amount of gamma globulin. The beta fraction was unaltered but the albumin and alpha fractions were relatively reduced. An example of the changes seen following thyroidectomy is shown in Fig. 1.

The concentration of total plasma hexosamine increased following thyroidectomy (middle part of Table I). This change could

‡ The dye-binding capacities of albumin and globulins differ from one another(7) so that the protein values are not those one would get with moving boundary electrophoresis, where albumin values run higher and globulins lower. It was shown in these experiments that the measurements used followed the Beer-Lambert law. This was established by scanning stained strips prepared by electrophoretically separating serum proteins of known concentration. It was shown that the areas (as determined by O. D. readings) under each band were proportional to the original concentrations of protein.

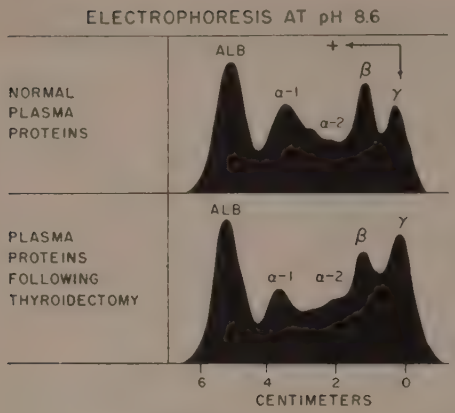

FIG. 1. Effect of thyroidectomy on plasma proteins of the rat.

be accounted for almost entirely by a significant increase in the hexosamine content of both the beta and gamma globulin fractions. The albumin hexosamine was also increased but this contributed little toward the total plasma hexosamine change. The alpha globulins were unaffected. With the known normal distribution of plasma proteins and hexosamine in plasma proteins (Table I), the concentration of hexosamine in each fraction can be estimated (assume a normal plasma protein concentration of 6%). As can be seen in the lower part of Table I, the alpha globulins were the richest hexosamine-containing proteins with albumin and gamma globulin having the lowest concentrations.

Discussion. Normal rat plasma contains all the major protein components seen in human plasma. Whereas human plasma normally contains more alpha-2 globulin, the reverse appears to be true in rat plasma. Many workers have reported difficulty or inability to demonstrate alpha globulins in normal rat serum with the use of moving boundary electrophoresis(8-13). Experience in our laboratory has shown that the separation of the alpha globulins by zone electrophoresis, although much slower with rat than with human plasma, will result with more time or higher voltage. It is likely that the reported absence of alpha globulins by Moore *et al.*(8,9) was in actuality a failure to separate albumin from alpha globulins. That rat plasma normally contains alpha globulins has clearly been demonstrated here and elsewhere(12).

The distribution of hexosamine in normal rat plasma proteins is similar to that seen in human serum(2), being richest in the alpha globulins(14,15).

The plasma mucoprotein fraction described by Winzler(16) is rich in hexosamine and migrates electrophoretically at pH 8.4 with alpha globulins(17). Mancini, Garberi, and de la Balze(18) have reported that in severe myxedema the mucoproteins, as determined chemically by Winzler's(16) method, are increased. On the other hand, Lewis and McCullagh(19) reported that the alpha-globulins are increased in hyperthyroidism and decreased in hypothyroidism. In support of this is the observation that plasma mucoproteins, as measured electrophoretically at pH 4.5, are increased in hyperthyroidism and decreased in hypothyroidism(20). These divergent observations are difficult to resolve without further study.

In the rat the alpha globulins do not appear to be altered following thyroidectomy. This is contrary to the observations of Moore, Levin, Smelser(9), who found that alpha globulins were absent in normal rats but appeared following thyroidectomy (see above), but is in agreement with the earlier findings of Levin and Leathem(3) that serum globulins in the rat, as measured chemically, increase following thyroidectomy. The findings here indicate that the greatest protein increase is in the gamma globulin fraction and that both the beta and gamma globulin fractions account for the increase in plasma hexosamine seen following thyroidectomy(1).

Since thyroidectomized rats do not develop the alpha-globulin changes which are seen in humans with hypothyroidism this probably represents a species difference.

Plasma fibrinogen is closely associated with, but poorly separated from, beta globulins(11) in the rat. It could not be clearly distinguished in the studies reported here.

Summary. Following thyroidectomy the rat develops a relative increase in gamma globulin. The increase in total plasma hexosamine levels following thyroidectomy can be

almost entirely accounted for by the increase in the hexosamine content of the beta and gamma globulin fractions. The normal distribution of hexosamine in rat plasma is as follows: Albumin 5%, alpha globulins 67%, beta globulins 19%, and gamma globulin 9%. The richest fraction is alpha-1 globulin which contains approximately 4.4% hexosamine. The other globulins contain less and albumin contains only 0.30%.

1. Boas, N. F., and Foley, J. B., *Endocrinology*, 1955, v56, 305.

2. Boas, N. F., and Reiner, M., *J. Clin. Endocrinol.*, 1951, v11, 890.

3. Levin, L., and Leathem, J. H., *Am. J. Physiol.*, 1942, v136, 306.

4. Boas, N. F., and Peterman, A. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 19.

5. Boas, N. F., *J. Biol. Chem.*, 1953, v204, 553.

6. Durrum, E. L., *J. Am. Chem. Soc.*, 1950, v72, 2943.

7. Kunkel, H. G., and Tiselius, A., *J. Gen. Phys.*, 1951, v35, 89.

8. Moore, D. H., Levin, L., and Leathem, J. H., *J. Biol. Chem.*, 1944, v153, 349.

9. Moore, D. H., Levin, L., and Smelser, G. K., *ibid.*, 1945, v157, 723.

10. Li, C. H., and Reinhardt, W. O., *ibid.*, 1947, v167, 487.

11. Deutsch, H. F., and Goodloe, M. B., *ibid.*, 1946, v161, 1.

12. Gjessing, E. C., and Chanutin, A., *ibid.*, 1947, v169, 657.

13. Jameson, E., and Alvarez-Tostado, C., PROC. SOC. EXP. BIOL. AND MED., 1939, v40, 476.

14. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.*, 1950, v185, 561.

15. Werner, I., and Odin, L., *Upsala Läkar, Förh.*, 1949, v54, 69.

16. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.

17. Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 110.

18. Mancini, R. E., Garberi, J. C., and de la Balze, F. A., *Rev. Soc. Argent. de Biol.*, 1951, v27, 285.

19. Lewis, L. A., and McCullagh, E. P., *Am. J. Med. Sc.*, 1944, v208, 727.

20. Mustacchi, P., Peterman, M. L., and Rall, J. E., *J. Clin. Endocrinol. and Metab.*, 1954, v14, 729.

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Stimulation of Adenine Uptake into Mouse Lung and Liver *in vitro* By Tumor Extracts.* (21923)

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Kelly and Jones(1) observed that repeated injections of tissue mashes into normal animals bring about changes in the nucleic acid turnover of the spleen and liver. Our studies (2) have indicated that single injections of a tumor homogenate into normal mice alter the incorporation of adenine-8-carbon^{14} (C^{14}) into the desoxyribonucleic acid (DNA) of several tissues. Fractions obtained from tumor homogenates by centrifugation or pH adjustment were tested for the presence of the factor(s) responsible for this effect by injection into normal mice simultaneously with adenine-8-C^{14} solution. Increase of lung DNA C^{14} activity in animals which had received the tumor preparations was taken as an indication that the factor(s) was present in the preparation. Goldwasser(3) reported that an *in vitro* system consisting of rat liver slices or of cell-free homogenates of pigeon liver incorporated adenine into ribonucleic acid but not into DNA. In simplifying the assay procedure for the tumor factor(s), the authors have developed an *in vitro* system in which significant increases in DNA C^{14} radioactivity

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after proper incubation with adenine-8-C^{14} have been observed.

Methods. The *in vitro* system consisted of a tissue mince prepared from the lungs and livers of normal 9- to 11-week-old C57 black mice.§ The lungs and livers were rapidly excised, weighed, placed on a watch glass, and minced together by scissors and scalpel until a finely divided mince of the combined tissues was obtained. Approximately equal portions of the mince were placed in wide mouth, 4-inch centrifuge tubes. Three to 5 ml of Ringer's solution fortified with glucose and buffered to pH 7.4 with a .01 ionic strength phosphate buffer was added to each tube. The tubes were maintained at 37°C in a water bath, and the contents of the tubes were continuously aerated by forcing air through long capillaries opening near the bottom of the solution in each tube. The same amount of adenine-8-C^{14} was then added to each tube, approximately 0.5 μc. Tumor homogenate of lyophilized tumor was added to the assay tubes. Other tubes, used as controls, were heated in boiling water for 5 minutes. Following a 4-hour incubation period, the contents of each tube were transferred to narrow centrifuge tubes, and the solid matter was spun down. The supernate was discarded, and the solid matter was washed twice in 10 ml of distilled water; the washings were also discarded. The remaining solids were weighed, transferred to Ten Broeck homogenizers, and homogenized in distilled water to 15 ml total volume. Five ml of the homogenate were taken for DNA extraction by the procedure of Schmidt and Thannhauser(4); other fractions extracted by this procedure were discarded. The DNA was dried on aluminum discs, and C^{14} radioactivity was determined by employing an automatic, windowless, gas-flow counter developed in this laboratory(5). Values of phosphorus concentration of the DNA were obtained utilizing the colorimetric method of King(6). Adenine-8-C^{14} was synthesized by the procedure of Clark and Kalckar(7). The tumor tissue added to assay tubes was obtained from transplantable mammary carcinoma E-0771. The tumor was palpable in 10 days and lethal in 30. Tumors were removed approximately 20 days after subcutaneous inoculation and were homogenized in Ten Broeck homogenizers.

To confirm these results, a series of experiments was carried out on liver and also on lung minces. Normal 5- to 10-week-old BAF^1 mice were used and mammary carcinoma E-0771 obtained from BAF^1 tumor bearing mice.§ The technic used was essentially the same as described above except for the following: exactly 4 ml of Ringer's glucose in phosphate buffer (pH 7.4, ionic strength .01) was used in each tube; stainless steel planchets were used for counting on an automatic Tracerlab Superscaler; and the Adenine-8-C^{14} used was obtained from Isotopes Specialties Co. The specific activity of the preparation was 1.93 mc/mM.

Results. The results are expressed in units of disintegrations/second gram wet weight of washed minced tissue. Values are also calculated in terms of disintegrations/sec./mg DNA phosphorus, and no significant differences between the 2 expressions were found. Duplicate values for each treatment were prepared where 2 values are given.

Groups 5, 6, and 7 were carried out under slightly different conditions and the 2 experiments should not be directly compared, but it is noteworthy that Group 5 falls within the range determined in Group 1.

That *in vitro* incorporation of adenine ac-

TABLE I. Values of Radioactivity for DNA Extracted after Incubation with Adenine-8-C^{14}.

Group	Treatment of tissue mince	Dis/sec/g wet wt of washed mince tissue
1.	Untreated	4.8, 7.5
2.	Tissue mince heated 5 min. in boiling water	.9, 1.2
3.	Tissue mince + 100-200 mg homogenized tumor tissue	8.3
4.	Tissue mince + lyophilized tumor homogenate corresponding to 400 mg tumor tissue	6.0, 8.9
5.	Untreated	5.5
6.	Tissue mince + 25 mg tumor homogenate	18.4
7.	Tissue mince + 50 mg tumor homogenate	16.2

§ C57 bl and BAF^1 mice obtained from the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Me.

TABLE II. Values of Radioactivity for DNA Extracted after Incubation with Adenine-8-C^{14}.

Group	Treatment of liver mince	Dis/sec/g wet wt of washed mince tissue
8.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue	3.5
9.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue (5 Mc adenine-8-C^{14})	25.4
10.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue	3.7
11.	Untreated	0
	Liver mince heated 5 min. in boiling water	0
	Liver mince + 25 mg homogenized tumor tissue	17.6

tually does occur is indicated by the decreased radioactivity in the tubes where the mince was deactivated by heat. The stimulating effect of tumor tissue on the uptake of adenine into DNA appeared variable, but gives further evidence that active uptake mechanism was operating *in vitro*. It was found in the earlier work referred to above(2) that stimulation of adenine uptake into DNA *in vivo* was also variable. In general, the variability was thought to be due to the lability of the factor(s) in solutions of tumor homogenate.

The results of Group 4, Table I, might indicate that some of the active principle in tumor homogenate can be preserved by lyophilization. The results of Groups 5 and 7 suggest that there may be a limit to the capacity to stimulate adenine uptake. In addition, they indicate that the increased radioactivity subsequent to the addition of tumor homogenate is not merely the effect of the added "active" DNA of the tumor cells, since, in that case, Group 7 should be much higher in value than Group 6.

It was not felt necessary to maintain strictly sterile conditions during the preparation and incubation of the tissue mince since the short incubation time was not adequate for extensive growth of microorganisms. Microbiological destruction or incorporation of adenine-8-C^{14} would have had to have varied from tube to tube to have seriously affected the results.

The results shown on Table II indicate that the same increase in the uptake of labeled adenine is obtained when tumor homogenate is added to a liver mince. This was verified in 4 studies. In no instance was this effect noted in lung minces. A final control study was carried out to ascertain the amount of uptake of adenine by tumor tissue. A tumor mince was prepared under the same conditions as the untreated and the heated controls as described above. The uptake by 25 mg of tumor mince was calculated and found to be 0.5 d/sec. while the heated tumor mince showed no uptake. Thus, the uptake by the amount of tumor added to the liver mince is only a small percentage of the total obtained for the liver plus the tumor, and we may assume that the radioactivity recorded represents uptake by the liver DNA fraction.

Summary. C57 bl mouse lung-liver mince and BAF[1] mouse liver mince incubated with adenine-8-C^{14} showed greater DNA activity than a similar system which had been deactivated by heat. This increased activity was not found with lung mince. A factor(s) present in tumor homogenate and possibly also in lyophilized tumor homogenate increased the DNA radioactivity to values significantly higher than those from tissues incubated without tumor homogenates.

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1. Kelly, L. S., and Jones, H. B., *Am. J. Physiol.*, 1953, v172, 575.

2. Furlong, N. B., Jr., Watson, E. J. P., and Davis, W. E., and Griffin, A. C., in preparation.

3. Goldwasser, E., *J. Biol. Chem.*, 1953, v202, 751.

4. Schmidt, G., and Thannhauser, J. S., *ibid.*, 1945, v161, 83.

5. Nye, W. N., and Teresi, J. D., *Anal. Chem.*, 1951, v23, 643.

6. King, E. J., *Biochem. J.*, 1932, v26, 292.

7. Clark, V. N., and Kalckar, H. N., *J. Chem. Soc.*, 1950, 1029.

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Renal Clearance of Para-Amino Salicylic Acid in the Dog.* (21924)

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(Introduced by Eric Ogden.)

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In comparing the renal clearances of para-amino salicylic acid (PAS) and inulin in man, Horne and Wilson(1) found that the former compound had a higher clearance than the latter: The excess clearance was depressed below the level of the inulin clearance and the plasma level of PAS elevated by the administration of caronamide. On the basis of these findings, it has been postulated that there is renal tubular excretion of PAS and a number of reports(2-4) have indicated that the administration of tubular blocking agents (caronamide and Benemid) will raise the level of plasma PAS in man. However, others(5-7) have failed to show persistent differences in plasma PAS levels after oral doses when Benemid is employed as a tubular blocking agent. In the present study we have investigated the renal clearance of PAS in the dog. Our results indicate that the clearance of PAS in this species is less than the glomerular filtration rate. We have furthermore found that Benemid does not depress the renal clearance of PAS.

Methods. Female dogs anesthetized with sodium pentobarbital (30 mg/kg intravenously) were used in all experiments. In prolonged PAS infusion experiments, supplementary anesthesia was given by including the barbiturate in the infusion in quantity sufficient to supply 3 mg/kg/hour.

For the determination of bladder clearances, a priming dose of 1.4-1.6 g each of sodium PAS (Parke-Davis Co.) and creatinine in 50 ml saline was followed by an infusion through a constant infusion syringe pump of the same solution at .5 ml/min. Adequate urine flow was secured by the administration of 20 ml of 10% sodium chloride solution. After a 10-30 minute period of equilibration, the bladder was washed with three 50 ml volumes of distilled water and the washings were analyzed for both PAS and creatinine. The clearance periods were then begun. Three consecutive clearance periods of 20 minutes each were run, arterial blood samples being taken at the beginning and end of each period. Aliquots of plasma and dilute urine were precipitated with cadmium hydroxide. PAS was determined on the supernate by the method of Newhouse and Klyne(8), and creatinine by the method of Bonsnes and Taussky(9). The color measurements were carried out on a Beckman DU spectrophotometer at 545 mu for creatinine. The recovery of each substance was estimated by adding a standard amount of both to blank plasma and comparing the color developed with that of the standard alone. Recovery of added PAS varied from dog to dog ranging from 63 to 99%. Duplicate determinations on any one sample, however, checked within 5%. Variable recovery of added PAS from plasma has been previously reported(8) but remains unexplained. Plasma concentrations were corrected for the PAS and creatinine recoveries. In the calculation of the bladder clearance of PAS, the plasma concentration was corrected for PAS binding. The original plasma concentration was multiplied by the ratio between ultrafilterable and total plasma PAS as determined on a separate blood sample for each dog. Plasma ultrafiltrates were made by subjecting whole blood in ¼ inch cellophane tubing to 15 lb air pressure. The ultrafiltrate was collected in two separate portions which were analyzed separately. The PAS concentrations of the ultrafiltrate did not change with time within the limits of the analytical method. In all bladder clearance experiments, the creatinine clearance was simultaneously measured. The plasma creatinine level was corrected for the small amount of creatinine binding by the use of the ultrafiltrate plasma creatinine factor.

The volumes of distribution of PAS and

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TABLE I. Renal Clearances and Volume of Distribution of PAS and Creatinine in the Dog (9 Animals).

Dog #	Wt, kg	Cl_{cr}, ml/kg/min.	Cl_{PAS}, ml/kg/min.	$\frac{Cl_{PAS}}{Cl_{cr}}$	V_{cr},* % B.W.	V_{PAS}, % B.W.	$\frac{V_{PAS}}{V_{cr}}$
1	9.6	4.2	3.1	.75			
2	9.5	3.9	3.4	.86			
3	11.4	3.3	2.9	.87			
4	20.0	4.8	3.7	.77			
5	18.0	4.1	3.8	.93			
6	15.4	2.1	1.6	.75	45	41	.90
7	13.0	3.7	3.5	.94	43	46	1.07
8	14.9	4.2	3.4	.82	44	44	1.00
9	15.5	5.0	2.9	.58	53	52	.99

* Volumes determined by the method of Greenberg *et al.*(10).

creatinine were estimated by the method of Greenberg *et al.*(10). From the total amount of PAS and creatinine administered in the primer and infusion, was subtracted the amount found in the bladder at the time that the first plasma sample was taken. The difference, representing the total amount of PAS and creatinine in the body was divided by the equilibrium concentration of the 2 substances in unbound form. No attempt was made to estimate the content of either substance in the renal dead space, so that these estimates of volumes of distribution were falsely high(11).

In experiments employing Benemid, the bladder clearance of PAS was determined as described above. After 3 consecutive clearance periods 0.5 g of Benemid dissolved in an equivalent amount of $NaHCO_3$(12) was injected intravenously in a 50 ml volume. The pH of the solution was 7.8. After a 10-minute period the PAS clearance was again determined in 3 consecutive periods.

Results. Binding of PAS by plasma proteins. In 12 measurements of the ratio of PAS concentration in the ultrafiltrate to that in the plasma, the free PAS was found to account for 49 to 88% of the total. The average value was 67% and the standard deviation was ± 9%. This variability in the protein binding has been noted previously in the case of other salicylates(13) and the binding of PAS in human blood is likewise variable (14).

Bladder clearance of PAS. In the first 5 bladder clearance experiments, the clearance was calculated from the plasma PAS concentration corrected by the average value for protein binding. In the last 4 experiments, the actual PAS binding by the individual dog's plasma was used in the calculation. The PAS clearance values ranged from 1.5-3.8 ml/kg/min. The creatinine clearances ranged from 2.0-5.0 ml/kg/min. The ratio of PAS to creatinine clearance ranged from .58 to .94 with an average of .81. In only 1 of 37 clearance periods did the ratio exceed 1. In this period the clearance ratio was 1.1.

Effect of Benemid on the bladder clearance of PAS. The bladder clearances of PAS in the 3 Benemid experiments were 20.8, 22.5, and 47.0 ml/min before the administration of Benemid and 30.7, 29.6 and 47.0 ml/min respectively afterwards. The creatinine clearance was unchanged in the first 2 experiments and rose slightly (from 65 to 76 ml/min) in the third.

Volumes of distribution of Creatinine and PAS. The volume of distribution calculated from the equilibrium plasma concentration and the PAS balance is, as has been noted, falsely high because of the PAS contained in the renal dead space. The average value obtained in experiments in which this method was employed was 45.5% of the body weight. In the same experiments, the volume of distribution of creatinine similarly calculated (and with almost the same error) was 46% of the body weight. We have noted elsewhere (11) that the true volume of distribution of creatinine as determined by disappearance curve analysis which eliminates the dead space error is closer to 35% of the body weight. Similar analyses of disappearance curves after single injections of PAS which

are not reported here yielded almost identical values for the volume of distribution of this substance (34% of the body weight). A similar value has been reported for the dog by Way *et al.*(14).

Discussion. The results demonstrate that in the dog the clearance of PAS is less than the glomerular filtration rate as measured by the clearance of creatinine. The fact that the clearance ratio is less than one may be interpreted to indicate that tubular reabsorption of PAS occurs in excess of tubular secretion of this molecule, if indeed the latter occurs at all. The possibility that secretion and reabsorption of PAS occur together appears unlikely because of the relatively narrow range of clearance ratios observed between PAS and creatinine. We were further unable to observe the diminution of PAS clearance with Benemid which would have been expected if this material interfered with the tubular secretion of PAS. In fact, in 2 of 3 experiments, Benemid caused an increase in the clearance of PAS.

The evidence for tubular secretion of PAS upon which the rationale of the use of tubular inhibitors is based is scanty. Only Horne and Wilson(1) appear to have compared PAS clearance and filtration rates. Others have confined their studies to the measurement of plasma levels after the oral administration of PAS with or without tubular inhibitors.

The present experiments indicate that in the dog at least, there is no evident tubular excretory activity toward PAS. If, in fact, plasma levels of PAS are elevated by such inhibitors, a finding which has been questioned repeatedly(5-7), it would appear that the site of action is not on the renal tubule.

Summary. Simultaneous measurements of the renal clearance of PAS and creatinine in the dog indicate that the former substance is not excreted by the renal tubules. Benemid does not appear to diminish the renal clearance of PAS in the dog. This raises question as to the rationale of employing Benemid as an adjunct to PAS therapy in man. The volume of distribution of PAS appears to correspond closely with that of creatinine.

1. Horne, N. W., and Wilson, W. M., *Lancet*, 1949, v257, 507.

2. Boger, W. P., and Pitts, F. W., *Science*, 1950, v112, 149.

3. Carr, D. T., Karlson, A. G., and Bridge, E. V., *Proc. Staff Mayo Clinic*, 1952, v27, 209.

4. Boger, W. P., Beatty, J. O., Pitts, F. W., and Flippin, H. F., *Ann. Int. Med.*, 1950, v33, 18

5. Rieber, C. W., Saline, M., and Freedman, M. M., *Am. Rev. Tuberc.*, 1951, v64, 448.

6. Riley, C., Clowater, R. A., and Shane, S. J., *Diseases of Chest*, 1953, v23, 90.

7. Rekola, J., *Acta Tuberc. Scand.*, 1953, v28, 113 (abstract).

8. Newhouse, J. P., and Klyne, W., *Biochem. J.*, 1949, v44, vii.

9. Bonsnes, R. W., and Taussky, H. H., *J. Biol. Chem.*, 1945, v158, 581.

10. Greenberg, J., Schwartz, I. L., Spinner, M., Silver, L., and Starr, N., *Am. J. Physiol.*, 1952, v168, 86.

11. Sapirstein, L. A., Vidt, D. G., Mandel, M. J., and Hanusek, G., *ibid.*, 1955, v181, 330.

12. Beyer, K. G., Russo, H. F., Tillson, E. K., Miller, A. K., Verwey, W. F., and Gass, S. R., *ibid.*, 1951, v166, 625.

13. Williams, F., and Leonards, J. R., *J. Pharm. Exp. Ther.*, 1948, v93, 401.

14. Way, E. L., Smith, P. K., Howie, D. L., Weiss, R., and Swanson, R., *ibid.*, 1948, v93, 368.

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Tissue Storage in Cadmium Treated Pigs. (21925)

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During an investigation of the ascaricidal activity of cadmium added to the feed of pigs (1,2), cadmium analyses of various tissues were made. The purpose was to determine the extent to which ingested cadmium was retained and to locate the tissues where greatest storage occurred. Although the above authors reported no evidence of toxicity with the dosage of cadmium used, it was considered desirable to see if tissue levels reached concentrations approaching those previously reported in animals showing definite signs of poisoning.

Method. Pigs, varying in weight from 25 to 150 lb, were fed Aska-Rid* in the diet for 72 hours. They were bathed prior to and following treatment and just before autopsy to prevent contamination of tissue samples. They were fed *ad libitum* during the experiment, and no reduction in food intake was noted for the treatment period. With the type ration used for administering cadmium, a 50-lb pig will eat about 2.5 lb per day(2). Thus, the cadmium oxide intake for 3 days would be 510 mg at the 68 mg per lb concentration. The first group received 68 mg cadmium oxide per lb of feed and were sacrificed at intervals of 14 and 30 days following treatment. This is the cadmium concentration recommended for worming pigs when used in a 3-day feeding program. The second group received 91 mg cadmium oxide per lb of feed and were sacrificed at intervals of 1, 3, 7, 14, 60, and 90 days following treatment. The third group received 68 mg cadmium oxide per lb of feed for 3 days. At the end of 6 weeks, they were again treated with feed containing 68 mg cadmium oxide per lb for 3 days. They were sacrificed 30 days after the second cadmium feeding. The fourth group received no cadmium and served as controls. Cadmium determinations were made in duplicate by the dithiozone method of Church(3).

Results. The highest concentration reported (Table) was 1.30 mg of cadmium oxide per 100 g of fresh tissue. This was from the spleen of a pig fed exclusively on 91 mg cadmium oxide per lb feed for 3 days and autopsied one day later.

Cadmium levels in the various tissues drop rapidly with only the kidney and spleen still showing significant increases as long as 14 days after the feeding experiment. Therefore, in subsequent determinations (30, 60, and 90 days after cadmium feeding), only these 2 tissues and the liver were analyzed. The liver was included, as others had reported high levels in this organ(3,4). When 2 feedings are made at a 6-weeks' interval, cadmium storage is greater than on a single feeding, but in no tissue was it as high as 1 mg per 100 g of fresh tissues 30 days after the second feeding.

Most studies reported on cadmium concentration in tissues are following administration by inhalation. Under such circumstances, the highest concentration is usually in the lungs. In fatal or near-fatal doses, this may be as high as 10 mg per g of dried lung tissue in the rat(4). On the other hand, cadmium oxide inhalation experiments in dogs(5) resulted in concentrations of cadmium oxide as high as 13.3 mg per 100 g of fresh kidney tissue. The highest in the liver was 3.9 mg. In these animals, toxicity was not noted nor were definite pathologic changes seen at autopsy. Prodan(6) fed 10 mg of cadmium per day to cats for 2 months. Emesis occurred occasionally during the first few days and ceased. Near the end of the experiment all animals manifested a decreased appetite. Tissue cadmium was 5.62 mg per 100 g of tissue for the liver, 4.65 mg for the kidney, and 1.11 mg for the spleen. Tipton(7) has recently assembled some very revealing data on cadmium con-

* Aska-Rid is the tradename for Pitman-Moore Co. brand of anthelmintic powder containing 1.5% cadmium oxide in an inert diluent.

TABLE I. Distribution of Cadmium in mg %.

Tissue	Untreated pigs	Treated pigs*								Repeat treatment† with 68 mg/lb CdO
		91 mg/lb CdO						68 mg/lb CdO		
		Days following treatment								
		1	3	7	14	60	90	14	30	30
Liver	.05(3)‡	.22	.12	.16	.16	.36(2)	.28(2)	.02(2)	.41(2)	.92(4)
Kidney	.03(3)	1.05	1.04	.53	.41	.23(2)	.16(2)	.38(2)	.27(2)	.65(4)
Spleen	.05(3)	1.30	.84	.91	.35	.46(2)	.24(2)	.05(2)	.53(2)	.92(4)
Blood	.02	.19	.25	.36	.13			.16(3)		
Brain	—	.97	.39	.09	.06			.02(2)		
Heart	—	.59	.25	.07	.00			.04(2)		
Muscle	—	.25	.17	.02	.04			.02(2)		
Stomach tissue	—	.12	.00	.53	.00			.16(2)		
" contents	—	.48	.06	.09	.00			.06(2)		
Ileum	—	1.10	—	—	—			—		
Cecum	—	1.23	.06	—	—					
Mid-jejunum	—	.80	.18	.26	.06			.32(2)		
Colon contents	—	—	—	—	—			.02(2)		
Rib bone	—	—	—	.10	.03			.04(2)		
Subcut. fat	.02	—	—	—	—			.11		
Abdominal fat	.02	—	—	—	—			.16		

* All received food containing indicated concentration of CdO for 3 days.
† Same dose repeated 6 wk after first; pigs sacrificed 30 days after second dose.
‡ No. in parentheses indicates No. of pigs from which average obtained. No number in parentheses indicates only tissue from one pig analyzed.

tent of human kidneys obtained at autopsy. On a fresh tissue basis, the average was 3.31 mg of cadmium per 100 g for kidneys and 0.36 mg for liver. There was no known exposure to cadmium in any of these subjects.

Summary. Feeding cadmium oxide-containing food for 3 days at concentrations of 68 mg and 91 mg per lb of food results in cadmium retention in the liver, spleen, and kidneys. The increase is significant when compared to controls but is very low when compared to cadmium levels others have reported by inhalation or feeding. Humans with no known exposure to cadmium have higher cadmium levels in the kidneys than pigs fed cadmium as described above.

1. Bunde, C. A., Blair, H. E., Burch, G. R., and Lee, J. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 547.

2. Burch, G. R., and Blair, H. E., *J. Am. Vet. M. A.*, 1955, v126, 304.

3. Church, F. W., *J. Indust. Hyg. and Toxicol.*, 1947, v29, 34.

4. Barrett, H. M., Irwin, D. A., and Semons, E., *ibid.*, 1947, v29, 279.

5. Princi, F., and Geever, E. F., *Arch. Indust. Hyg. and Occup. Med.*, 1950, v1, 651.

6. Prodan, L., *J. Indust. Hyg. and Toxicol.*, 1932, v14, 174.

7. Tipton, I. H., Foland, W. D., Bobb, F. C., and McCorkle, W. C., personal communication to Dr. C. A. Bunde.

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Synthesis of Glycogen Fractions by Heart Homogenates.* (21926)

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Glycogen synthesis, *in vitro,* has been investigated in a number of tissues. Hastings (1) reported synthesis in liver, Stadie and Zapp(2) in diaphragm, Stadie, Haugaard and Perlmutter(3) in heart and Marsh and Miller (4) in the kidney. Interest in the glycogen fractions was renewed by the work of Bloom, Lewis, Schumpert and Shen(5), who described a method for the isolation of 2 types of glycogen through differential solubility in cold trichloroacetic acid (TCA). Cordier and Dessaux(6), utilizing this technic in an investigation of cardiac glycogen disappearance in rats subjected to anoxia, found that the TCA soluble fraction decreased to a greater extent than the TCA insoluble fraction of glycogen. A pronounced species variation of these 2 forms of cardiac glycogen was reported by Merrick and Meyer(7), who found in studies on dogs exposed to conditions of fulminating anoxia that although the TCA soluble glycogen disappeared rapidly from the heart, there appeared to be a temporary increase of the TCA insoluble fraction. The problem of changes in the cardiac glycogen fractions is worthy of more detailed analysis. The present study deals with the changes of concentration of the cardiac glycogens in rat and dog hearts, which have been homogenized in a medium promoting glycogen synthesis, and exposed to either an atmosphere of oxygen or an atmosphere of nitrogen.

Methods. For each of the 10 runs in each series of analyses, either the pooled ventricles from 6 decapitated male albino rats or a portion of the left ventricle from a dog anesthetized with pentobarbital sodium (32.5 mg/kg) was removed, weighed and then frozen between blocks of dry ice. Enough cold (1°C) fluid medium (Krebs Phosphate Ringer's solution or 5.4% glucose solution) was measured to make a 10% weight/volume mixture when homogenized with the heart tissue. The ventricles were ground with the fluid medium in a Ten Broeck Tissue Grinder, the temperature being held at approximately 2-3°C during homogenization. A 3 ml sample of the homogenate was taken for the initial glycogen value. The remainder of the homogenate was transferred to Warburg flasks, 3 ml per flask. The flasks were placed in a Warburg bath set for a temperature of 37.5°C, flushed with O_2 or N_2 and allowed to equilibrate for 30 minutes before oxygen consumption was recorded. One flask of each series of 7 was taken from the bath at succeeding time intervals (as indicated in Table I) and the contents removed for glycogen analysis. The final sample was removed 105 minutes after the initial sample. Extraction of the 2 glycogens was accomplished with essentially the procedure outlined by Bloom, *et al.*(5), that is, the TCA soluble fraction was extracted with cold TCA and separated from the precipitate by centrifugation at 1°C. One modification of the procedure, however, consisted of digesting the precipitate from the TCA extraction with 30% KOH to secure the non-extractable glycogen rather than using a duplicate sample and determining total glycogen by KOH digestion. After hydrolysis in sulfuric acid, the isolated glycogen samples were measured quantitatively by the anthrone procedure of Seifter, Dayton, Novic and Muntwyler(8).

Results and discussion. There was a precipitous drop of both glycogen fractions (Table I) which shows that glycolysis rapidly reduces heart glycogen when the organ has been homogenized. In contrast to the findings of Cordier and Dessaux(6) with the heart of anoxic rats (*in vivo*) where the TCA insoluble fraction was relatively stable, both fractions dropped to about 50% of their initial value within 5 minutes. Although no de-

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TABLE I. Glycogen Content of Heart Homogenates as Mean ± Standard Deviation.

Animal and medium	Glycogen	Time in min. after heart was homogenized					
		0	5	15	22½	30	60	75	105
Rat	TCA soluble	131 ± 37.7	66 ± 15.6	55 ± 8.8	49 ± 9.6	63 ± 19.3	79 ± 31.6	92 ± 38.5	103 ± 30.7
5.4% glucose	TCA insoluble	140 ± 44.9	74 ± 24.9	33 ± 15.8	23 ± 10.7	20 ± 7.4	16 ± 6.6	16 ± 5.4	22 ± 4.4
100% oxygen	Total	271	140	88	77	83	95	108	125
Rat	TCA soluble	154 ± 51.2	—	—	—	51 ± 22.5	54 ± 29.9	57 ± 38.2	54 ± 18.8
5.4% glucose	TCA insoluble	102 ± 37.7	—	—	—	19 ± 6.4	18 ± 3.7	15 ± 3.7	14 ± 2.6
3% oxygen	Total	256	—	—	—	70	72	72	68
97% nitrogen									
Rat	TCA soluble	86 ± 45.0	—	—	—	52 ± 43.9	53 ± 41.3	46 ± 32.7	34 ± 16.5*
Krebs phosphate	TCA insoluble	109 ± 54.7	—	—	—	25 ± 7.0	25 ± 11.9	23 ± 9.9	21 ± 6.5*
Ringer's	Total	195	—	—	—	77	78	69	55
1% glucose									
100% oxygen									
Dog	TCA soluble	471 ± 126.8	405 ± 165.3	244 ± 95.4	166 ± 52.7	125 ± 41.5	55 ± 14.8	45 ± 5.8	45 ± 5.9
5.4% glucose	TCA insoluble	114 ± 28.6	105 ± 16.3	90 ± 16.7	85 ± 20.1	80 ± 20.1	40 ± 16.8	25 ± 9.8	13 ± 2.9
100% oxygen	Total	585	510	334	251	205	95	70	58

* 90 min. samples. Each value represents the mean of 10 determinations.

terminations were made during the first half hour in the series utilizing Krebs Ringer's or low oxygen in 5.4% glucose, the 30-minute values suggest that they follow the same glycolytic pattern. A plateau independent of initial glycogen concentration is reached at 30 minutes. The TCA soluble glycogen, except when synthesis occurs, stabilizes at approximately 50 mg per 100 g of tissue while that part not extracted by cold TCA drops to 20 mg per 100 g of tissue.

This behavior of rat heart glycogen contrasts strongly with the situation in the dog where the TCA insoluble glycogen is found to decrease gradually over the 105 minute period (Table I). The TCA soluble glycogen of the dog heart, although disappearing rapidly, does not reach a stable level until 75 minutes after the first sample is taken.

Synthesis of glycogen by the rat heart was achieved in oxygenated 5.4% glucose confirming the experience of Stadie, Haugaard and Perlmutter(3). The most marked synthesis was in the TCA extractable portion, which after declining to 54 mg per 100 g of tissue, then began to rise and continued until a value of 103 mg per 100 g of tissue was reached at the end of 105 minutes. This represents synthesis at the rate of 0.594 mg of glycogen per 100 g of heart tissue per minute. The TCA insoluble glycogen, however, continued to decline for 75 minutes and then rose from 16 to 22 mg per 100 g of heart tissue at 105 minutes, a difference significant to the 98% confidence level. The insoluble fraction is resynthesized at roughly one-third the rate that the TCA soluble glycogen is formed.

Nothing in the data from rat heart homogenates suggests that the TCA soluble glycogen undergoes glycolysis in preference to the TCA insoluble fraction, although this is the implication from the work of Cordier and Dessaux(6) on the intact animal. However, this does seem to be true of the homogenized dog heart. The homogenization of the tissue may well alter metabolic pathways by destroying the normal anatomy of the cell.

The resynthesis of glycogen, under the conditions of the experiment, starts with the formation of the TCA soluble moiety. Whether or not the TCA insoluble fraction is

formed directly from the glucose, or represents the binding of the soluble form to proteins or some other molecule which is insoluble in cold TCA has yet to be determined.

The dog heart homogenate did not synthesize either glycogen fraction under the same conditions in which the rat heart showed glycogenic activity. Neither form of glycogen of rat heart was synthesized in Krebs Ringer's, though adequate glucose was present, nor in 5.4% glucose when oxygen tension was reduced to 21.5 mm Hg.

Glycogen synthesis was well correlated with oxygen consumption of the tissue homogenates. In the one medium (oxygenated 5.4% glucose) which promoted glycogenesis, oxygen consumption was 303 μl-O_2 per g of wet tissue per hour compared to the utilization of 145 μl-O_2 per g of wet tissue per hour in the unoxygenated sugar medium and the 131 μl-O_2 per g of wet tissue per hour in the oxygenated Krebs Ringer's. The dog heart suspension exhibited the lowest oxygen uptake, using only 114 μl-O_2 per g of wet tissue per hour.

Summary. Glycolysis reduces both glycogen fractions of the rat heart at about the same rate for the first 5 minutes, regardless of the medium used for suspension of the tissue. In Krebs Phosphate Ringer's solution and in unoxygenated 5.4% glucose, the 2 glycogens drop to a low, but stable level. The glycogen synthesized in the sugar medium is primarily the TCA soluble glycogen. The TCA soluble glycogen begins synthesis at the end of 22.5 minutes and synthesis of the TCA insoluble fraction begins 50 minutes later. A distinct species difference was observed in the metabolism of cardiac glycogen in the rat and dog, inasmuch as the TCA insoluble fraction was relatively more stable in the dog.

1. Hastings, A. B., *Harvey Lectures,* 1940-41, v36, 91.

2. Stadie, W. C., and Zapp, J. A., *J. Biol. Chem.,* 1947, v170, 55.

3. Stadie, W. C., Haugaard, N., and Perlmutter, M., *ibid.,* 1947, v171, 419.

4. Marsh, J. B., and Miller, K. L., *Science,* 1953, v118, 416.

5. Bloom, W. L., Lewis, G. T., Schumpert, M. Z., and Shen, T., *J. Biol. Chem.,* 1951, v188, 631.

6. Cordier, D., and Dessaux, G., *J. Physiologie,* 1952, v44, 703.

7. Merrick, A. W., and Meyer, D. K., *Am. J. Physiol.,* 1954, v177, 441.

8. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E., *Arch. Biochem.,* 1950, v25, 191.

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Clotting Factor X. Physiologic and Physico-Chemical Properties. (21927)

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The use of serum from various pathological conditions in the thromboplastin generation test(1,2) led to the conclusion that besides the serum factors already known (Factor VII = SPCA, proconvertin and Factor IX = Christmas factor, PTC),* another clotting factor exists in serum for which the designation Factor X(3-5) was proposed. Matching experiments with serum from hemophilia B (deficient in Factor IX) proved to be especially demonstrative.

Method. A modification of the thromboplastin generation test developed in our laboratory was employed(2). The 4 components incubated to generate blood thromboplastin are: Platelet suspension prepared according to Flückiger *et al.*(6), calcium chloride, oxalated plasma adsorbed on barium sulfate (source of antihemophilic globulin) and serum. The first 3 components are kept

* The following numbering of clotting factors is proposed: Fibrinogen (F.I), Prothrombin (F.II), Thromboplastin (F.III), Calcium (F.IV), Labile factor, Proaccelerin (F.V & VI), SPCA Proconvertin (F.VII), antihemophilic Globulin (F.VIII), P.T.C., Christmas Factor (F.IX).

constant (normal platelets and oxalated plasma being used), whereas serum is varied. The results of the test are given in incubation and clotting-times directly measured (not in thromboplastin-units). To study the mixture of 2 sera, both are diluted 1/10 with buffer, then 0.15 cc of each dilution added to the mixture of platelet suspension, adsorbed plasma and $CaCl_2$. *Quantitative determination of Factor VII*: according to Koller, Loeliger and Duckert(7). *Chloroform extract of human brain thromboplastin.* Acetone dried brain powder (1 g) is suspended in chloroform at room temp. 20 min, then filtered and washed with chloroform, (final vol. 50 cc). After evaporation of solvent at 50°C the residue is suspended in 50 cc physiological saline and stored at 30°C. This stock solution is diluted 1/100 with veronal-acetate buffer before use in the thromboplastin generation test, replacing the thrombocytes.

Purification of Factor IX and X. All operations at room temperature. *a*). *Purification of Factor IX by chromatography.* To prepare the column, 1 g of barium sulfate and 1 g of hyflosupercel are suspended in physiological saline, pH 7, and introduced into the column. Excess saline is discarded. Normal citrated serum (1.8 cc serum + 0.2 cc trisodium citrate 0.2 M) is poured on top of column. After the serum 0.5 cc saline is added and elution performed by means of trisodium citrate, 0.14 M. pH 7.8. Flowing speed is slow (1 drop/25 sec.). The eluate is fractionated into portions of 1 cc. The first fractions are rich in Factor VII. Factor IX appears in the fourth fraction. By chromatography Factor IX is obtained free of Factor X which is not eluted by this method. The fraction contains however 5 to 35% of Factor VII. *b*). *Purification of Factor IX by adsorption.* 10 cc of old normal serum (stored at room temperature a few days in order to remove Factor X) and 0.8 cc trisodium citrate 0.2 M (final concentration 0.0148 M) are mixed with 600 mg barium sulfate and stirred 5 min. with a glass rod. After centrifugation, the barium sulfate is washed with 2 cc distilled water and the supernatant discarded. Elution: the barium sulfate is suspended in 1 cc of trisodium citrate 0.14 M,

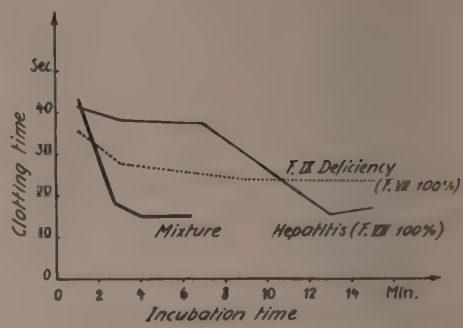

FIG. 1. Normalisation of thromboplastin formation with a mixture of hemophilia B-Serum (F. IX-deficiency) and hepatitis-serum.

pH 7.8, stirred 5 min. and centrifuged. The supernatant solution contains Factor IX. traces of Factor VII and is Factor X-free. *c*). *Purification of Factor X by adsorption.* 4.5 cc normal serum + 0.5 cc trisodium citrate 0.2 M (final concentration 0.02 M) are mixed with 250 mg barium sulfate and stirred 5 min. After centrifugation the supernatant is discarded, barium sulfate washed with 2 cc distilled water and the solution discarded. Elution: barium sulfate is suspended in 1 cc of trisodium citrate 0.14 M and stirred 3 min. The solution contains Factor X, traces of Factor VII and is Factor IX-free. To exclude any contamination with Factor IX this preparation may be made with hemophilia B-serum.

Results. Two types of abnormal thromboplastin generation curves. (Fig. 1) Normal serum yields a curve with minimal clotting time of 11 to 12 seconds after incubation time of 3 to 8 minutes. Serum from hemophilia B (deficient in Factor IX) gives a markedly prolonged minimal clotting time, reached however after a normal or almost normal incubation time. On the other hand serum from a patient treated with 3-(1-phenyl-propyl)-4-hydroxycoumarin (= Marcoumar), or from a patient with hepatitis (Fig. 1) produces a greatly delayed curve (incubation time over 12 minutes), but the clotting time finally reached is within normal limits. This curve corresponds to Factor X-deficiency. The difference in shape of the curve is even more pronounced when instead of platelet suspension a

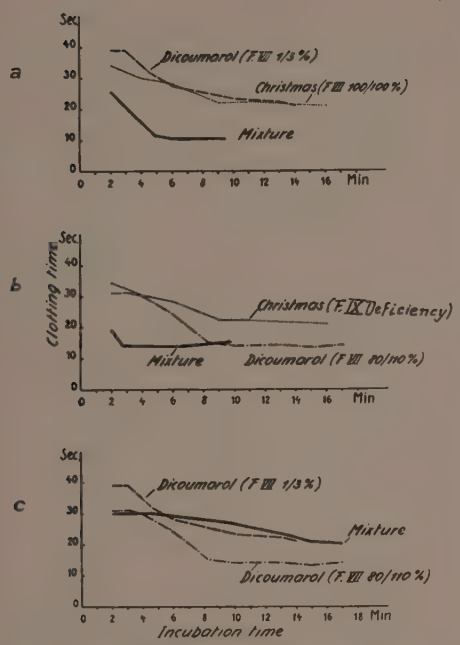

FIG. 2. Effect of a dicumarol derivative (Marcoumar) on thromboplastin formation. (Factor VII activity is given in plasma and serum.) In Exp. C, the concentration of Factor IX is even higher in the mixture than in assay a and b (in Christmas-serum Factor IX is very low), and the concentration of Factor VII is the same as in assay a. Nevertheless thromboplastin formation of the mixture is very poor in c but normal in a and b. A 3rd serum factor has to be assumed.

chloroform extract of brain is used in the generation test. If the concentration of Factor X is very low, thromboplastin formation is almost impossible. Therefore the clotting time (reciprocal to amount of thromboplastin formed) remains high as in Factor IX-deficiency, in these extreme cases(13).

Differentiation of Factor X from Factor VII and IX by matching experiments. A mixture of equal parts of the above mentioned Marcoumar-serum and hemophilia B-serum yields an entirely normal thromboplastin generation. (Fig. 2a). This finding might be in agreement with the hypothesis that insufficient thromboplastin generation of Marcoumar-serum is due to Factor VII-deficiency while that of hemophilia B-serum is caused by lack of Factor IX. However the following observation contradicts this view: Serum of a patient who had first been given Marcoumar, then, because of bleeding, vit. K_1, and whose Factor VII concentration had returned to normal (100% in plasma and in serum) produced a markedly delayed thromboplastin generation. Hemophilia B-serum normalises this clotting defect. (Fig. 2b). Therefore lack of Factor IX cannot account for the delay of thromboplastin generation, nor can Factor VII-deficiency do so. A third serum-factor (Factor X) must be involved. If the 2 Marcoumar sera (with 10 resp. 100% Factor VII) are mixed, there is no normalisation of thromboplastin generation. (Fig. 2c) Serum of epidemic hepatitis, of hepatic cirrhosis, of the new-born behaves in the same way as Marcoumar-serum. (Table I). Stored serum is also abnormal in thromboplastin generation although the concentration of Factor VII and IX remain unchanged during several days at room temperature. After 8 days of storage Factor VII 160% and Factor IX were normal. The delayed thromboplastin formation is therefore due to deficiency of another serum factor.

Purified Factor IX, mixed with hemophilia B-serum produces a normal thromboplastin generation; the same is true with purified Factor X when mixed with Marcoumar-serum. Matching of both purified preparations (Factor IX and X) gives a normal thromboplastin generation in spite of the very low Factor VII content of the mixture. The chromatographic fraction containing no factor X produces a very delayed thromboplastin generation when mixed with Marcoumar-serum.

Objections to these matching experiments. Hemophilia B-serum used in most matching experiments (Table I) contains considerable amounts of prothrombin, as a consequence of the reduced prothrombin consumption. During the thromboplastin generation test prothrombin is converted into thrombin and therefore may influence the results. Isenschmid(9), using fibrinogen as substrate instead of calcium-free plasma, was able to measure the amount of thrombin formed simultaneously with thromboplastin generation. This amount was substracted from the total quantity of thrombin measured with calcium-free plasma as substrate. In this way a "cor-

TABLE I. Matching Experiments.*

No.	Serum I	F. VII	IX	X	Serum II	F. VII	IX	X	Mixture† F. VII	IX	X	Thromboplastin generation	
		%				%			%				
1.	Marcoumar	3	+	—	Hemophilia B	100	—	+	50	—	+	normal	Fig. 2a
2.	Marcoumar + vit. K1	110	+	—	"	100	—	+	105	+	+	"	Fig. 2b
3.	Hepatitis, mild	100	+	—	"	100	—	+	100	+	+	"	Fig. 3
4.	*Idem*	100	+	—	Marcoumar	40	+	—	70	+		delayed	
5.	Hepatic cirrhosis	75	+	—	Hemophilia B	100	—	+	90	+	+	normal	
6.	New-born	45	+	—	"	100	—	+	75	+	+	"	
7.	Stored normal serum (8 days)	160	+	—	"	100	—	+	130	+	+	"	
8.	Factor IX purif. chromat.	35	+	—	"	100	—	+	70	+	+	"	
9.	*Idem*	35	+	—	Marcoumar	25	+	—	30	+	—	delayed	
10.	Factor IX purif. adsorpt.	7	+	—	Hemophilia B	100	—	+	55	+	+	normal	
11.	*Idem*	7	+	—	Marcoumar	50	+	—	20	+		delayed	
12.	Factor X purif. adsorpt.	7	—	+	"	35	+	—	20	+	+	normal	
13.	Factor X purif.	7	—	+	Hemophilia B	100	—	+	55	—	+	abnormal	
14.	Factor X purif. adsorpt.	7	—	+	Factor IX purif. adsorpt.	7	+	—	7	+	+	normal	

* All sera I and II used alone, produce an abnormal thromboplastin generation; Factor IX and X can be determined only approximately; + means normal, — means diminished or lacking activity in thromboplastin generation test; "purif. chromat." means purified by chromatography; "purif. adsorpt." means purified by adsorption.

† In a mixture of 2 sera resulting in normalisation of thromboplastin generation Factor IX and X concentrations do not exceed 50% of normal values.

As example of normalisation see Fig. 1, 2a and b. As example of non-normalisation see Fig. 2c.

rected" curve was obtained, which showed that normalisation of thromboplastin generation, by mixing Marcoumar- and hemophilia B-serum, persists. Only the "over-normalisation" has to be attributed to the prothrombin content of hemophilia B-serum. Isenschmid could demonstrate that thrombin has no accelerating effect on thromboplastin formation; therefore simple substraction of its effect is allowed. Another objection is furnished by the observation that matching of Marcoumar- and hemophilia B-serum does not always produce a complete normalisation of thromboplastin generation. In fact Dicoumarol and its derivatives although they lower primarily Factor X (besides prothrombin and Factor VII), affect also, to a lesser degree, Factor IX. Marcoumar, (the Dicoumarol-derivative we used most), lowers first the concentration of Factor X alone, and after a week or 2 the activity of Factor IX. Bis-3, 3'-(4-hydroxycoumarinyl) - ethyl - acetate (tromexan), on the other hand, seems to lower Factor IX more rapidly. It is evident that hemophilia B-serum cannot normalise the clotting defect of a serum simultaneously deficient in Factor IX and X. It has been suggested that a factor-X deficiency is a slight deficiency of Factor IX. This opinion is in contradiction to the results obtained by dilution of normal serum(2) where both incubation and coagulation time are prolonged, not incubation time alone. The assays with purified factors (Table I, No. 8-14) also contradicts this opinion as well as the experiments illustrated in Fig. 2. Another objection was that an excess of *antihemophilic globulin* might explain normalisation produced by mixing hemophilia B-serum with various pathological sera (Table I). We increased the concentration of antihemophilic globulin in the incubation mixture 5 times (by using a 1:5 instead of a 1:25 dilution of $BaSO_4$-plasma) without obtaining a normalisation of throm-

boplastin generation with hemophilia B or Marcoumar-serum. It is evident that excess of one clotting factor can compensate *partially* for the deficiency of another factor. A complete normalisation however has never been achieved by this procedure.

Insignificant role of Factor VII in blood thromboplastin formation. In Table I it may be seen (No. 14) that thromboplastin generation of the mixture is normal with as low a Factor VII content as 7% (100% Factor VII = normal concentration in plasma) whereas it is delayed with a concentration of 70% (No. 4 of Table I). A rise of Factor VII from 10 to 110% under the influence of Vit. K does not change significantly the delayed thromboplastin formation (Serum I in No. 1 & 2 of Table I). On the other hand a decrease of Factor VII in serum from 150% to 18% in the beginning of Marcoumar-treatment did not delay thromboplastin formation. It may therefore be concluded that Factor VII has very little, if any, influence in blood thromboplastin generation. (See also Fig. 2).

Physiological properties of Factor X compared with those of Factor IX. Incubation mixtures are prepared with constant amounts of all factors necessary for thromboplastin formation except Factor X, the concentration of which is varied from 1 to 100%. As seen in Fig. 3 the speed of thromboplastin formation is greatly enhanced by increasing amounts of Factor X, whereas the *amount* of thromboplastin produced (*i.e.* the clotting time finally reached) is practically unchanged. If in the incubation mixture the concentration of Factor IX is varied while all other factors are kept constant, the clotting time (corresponding to amount of thromboplastin formed) is primarily influenced (Fig. 4), whereas incubation time differs only slightly. It seems therefore that Factor IX is a precursor of blood thromboplastin and that Factor X has to be considered as an *accelerator of blood thromboplastin formation.* There is another difference between the 2 factors: in Factor IX-deficiency prothrombin consumption is markedly reduced whereas in "pure" Factor X-deficiency it is normal.

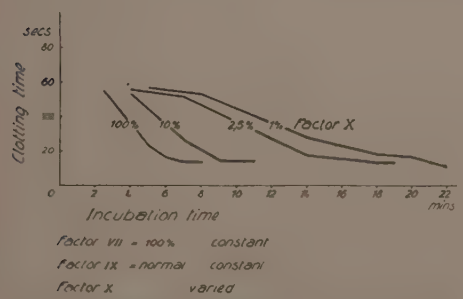

FIG. 3. Influence of variable Factor X concentrations, all other factors being kept constant. Clotting time normal, incubation time prolonged.

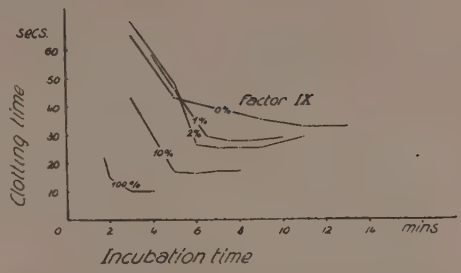

FIG. 4. Influence of variable Factor IX concentrations, all other factors being kept constant. Incubation time in normal range, clotting time prolonged.

Physico-chemical properties of Factor X. The properties of Factor X are almost identical with those of serum factors VII and IX. However its resistance to storage is lower. It disappears from normal serum in a few hours or a few days at room temperature. It is denatured at 50°C. Its affinity to $BaSO_4$ is more pronounced than that of Factor VII and IX (see method of purification). By chromatography it is irreversibly adsorbed. It is also adsorbed by $BaCO_3$ and $Ca_3(PO_4)_2$. In diluted solution (serum 1/10 with veronal acetate buffer pH 7.35) it is moderately activated at room temperature within 20 to 30 min.

Factor X compared to other recently described clotting factors. Factor X is not identical with PTA(10), the latter being only slightly adsorbed on barium sulfate. Deficiency of PTA produces an abnormal thromboplastin generation only if both $BaSO_4$ plasma and serum of the patient are used in the test. PTA is therefore neither adsorbed on $BaSO_4$ nor consumed during coagulation.

Two new clotting factors described by Spaet(11) and Ratnoff(12) are not identical with factor X because they are not adsorbed on barium sulfate.

Summary. 1. A new clotting factor, factor X, participating in blood thromboplastin formation is demonstrated by matching experiments with various pathological sera. The possible influence of thrombin and factor VII is eliminated. The objection that factor X deficiency may be nothing else than a slight deficiency of factor IX is discussed. This hypothesis is in contradiction with our results. 2. Factor X is present in normal serum and in hemophilia B-Serum, absent in hepatitis-serum and in serum of Marcoumar-(Dicoumarol derivative) treated patients. The affinity of factor X to barium sulfate is greater than that of Factor VII and IX. This property is utilised for the purification of Factor X. 3. Factor X is not identical with PTA and the new factors of Spaet *et al.* and of Ratnoff *et al.*

1. Biggs, R., Douglas, A. S., and Macfarlane, R. G., *Physiol.*, 1953, v119, 89.

2. Duckert, F., Flückiger, P., Isenschmid, H., Matter, M., Vogel-Meng, J., and Koller, F., *Acta haemat.*, 1954, v12, 197.

3. Koller, F., *Arch. exp. Path. u. Pharmak.*, 1954, v222, 89.

4. Flückiger, P., Duckert, F., and Koller, F., *Schweiz. Med. Wschr.*, 1954, v84, 1127.

5. Duckert, F., Flückiger, P., and Koller, F., *Rev. Hematol.*, 1954, v9, 489.

6. Flückiger, P., Hässig, A., and Koller, F., *Acta haematol.*, 1954, v12, 339.

7. Koller, F., Loeliger, A., and Duckert, F., *ibid.*, 1951, v6, 1.

8. Bell, W. N., and Alton, H. G., *Nature*, 1954, v174, 880.

9. Isenschmid, H., *Acta haemat.*, 1955, v13, 177.

10. Rosenthal, R. L., *Fed. Proc.*, 1954, v13, 284.

11. Spaet, T. H., Aggeler, P. M., and Kinsell, B. G., *J. Clin. Invest.*, 1954, v33, 1095.

12. Ratnoff, O. D., and Copley, J. E., *ibid.*, 1955, v34, 602.

13. Duckert, F., *Schweiz. Med. Wschr.*, in press.

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Response of Visual Area to Callosal Impulses in the Cat.* (21928)

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There are still uncertainties concerning the collosal connections of the visual area. The anatomical(1-3) and the oscillographic data are equivocal. Reactions of the area to a synchronized volley of callosal impulses have been found in the cat by Curtis(3) and also (from his experimental map) by Garol(4). But Curtis(3), McCulloch and Garol(5), Bailey, Bonin and McCulloch(6) were unable to discover any commissural connections from area 17 in the cat, the monkey and in the chimpanzee.

In the unanesthetized cat ("encéphale isolé" préparation) callosal responses of the visual area, which can be recorded with great regularity, have revealed special features which seem of interest.

Methods. All observations have been done on adult cats after an initial ether narcosis during which the spinal cord had been cut at the C_1 level, the optic nerve prepared on one side and the cortex exposed on both sides. The callosal volley was produced by a thyratron shock of 0.5 millisecond duration applied to a point of the contralateral gyrus lateralis symmetrical to the point whose potentials were recorded. The responses of various other areas were displayed simultaneously on the second beam of the cathode ray oscilloscope. The differential amplifier systems had an overall time-constant of 0.1 sec. The cortical stimulation was bipolar, the recording of the potentials monopolar. The response of the visual area I to shocks on the contra-

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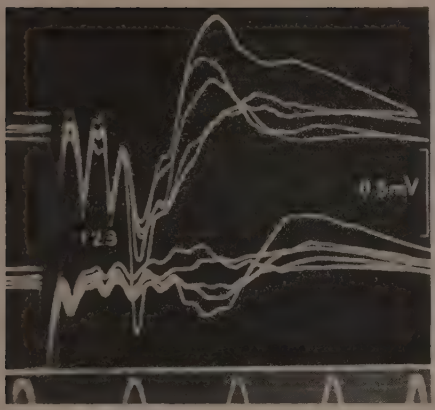

FIG. 1. A: Responses of area I (upper traces) and of suprasylvian gyrus to a shock on contralateral optic nerve (negativity upwards); 5 successive sweeps taken at 10 second intervals show lability of the slow phases of the response, contrasting with the stability of its spike-like components; time marks: 10 msec.

lateral optic nerve or to brief flashes of light were recorded for comparison.

Results. It seems opportune to recall first the characteristic pattern of the reaction of the visual area I to a volley of geniculo-cortical impulses. As described by Bartley and Bishop(7), the distinctive feature of the response is its beginning by a succession of surface-positive spike-like deflections, labeled 1, 2 and 3 in Fig. 1 (see also Figs. 2A, 3B), preceding the slower diphasic oscillation (positive-negative) which constitutes the reaction of all isocortical areas to a volley of afferent impulses (see Fig. 2B). Our own analysis has led us to endorse the conclusion of Chang and Kaada(8), but not confirmed by Bishop(9), that these initial spike-like deflections all represent radiation potentials assignable to the activities of different systems of geniculo-cortical neurones. As such these components of the response are much less labile than the following slower positive-negative complex which expresses the post-synaptic reaction of cortical neurones (Fig. 1, upper traces). This typical pattern is observed in all divisions of visual area I, with an eventual variation of amplitude of its components (Fig. 2A). Paravisual areas (Fig. 1, lower traces) show small dips in phase with the spikes of the visual area and probably representing the field effects of radiation potentials.

The recording of the response of the same visual area to a callosal volley revealed an analogous pattern to the sensory reaction just described, and quite unlike the callosal response of other cortical regions (Fig. 2C) for the acoustic area. Like the sensory response it is characterized by an initial succession of spike-like deflections preceding the slower potential and encroaching upon it. Callosal response from 3 different experiments is reproduced in Fig. 3B, C (upper trace) and D. The analogy is particularly striking between the lower trace of Fig. 2A and the upper trace of Fig. 3C because of the small amplitude of the slow components of the responses in both cases.

Except for small field effects (Fig. 3C, lower trace), the typical response is limited to the visual area. It is completely abol-

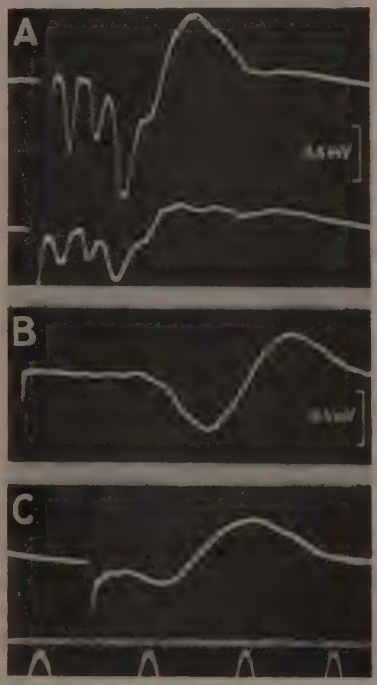

FIG. 2. A: Responses of middle portion of visual area (upper trace) and of posterior portion to optic nerve shock; B: auditory area I, response to a click; C: auditory area I, response to a shock on symmetrical point of opposite hemisphere; time: 10 msec.

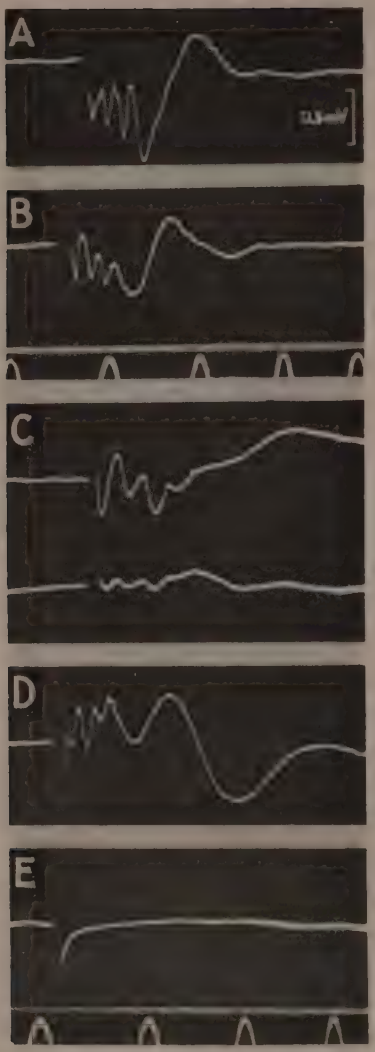

FIG. 3. A: Callosal response of visual area I; B: same cat, response of same point of visual area to optic nerve shock; C: another cat, callosal response of visual area I (upper trace) and of suprasylvian gyrus. D and E, another cat; D: callosal response of visual area I; E: complete disappearance of response after section of corpus callosum.

ished by the section of the corpus callosum (Fig. E) and it can exert on the sensory response (to an optic-nerve-shock or to a flash) following it the same facilitatory effect which has been described for the acoustic area(10,11). The precession of a callosal volley may treble the voltage of the sensory response. As for the acoustic area, the facilitatory modification is not related to any slow wave following the callosal volley and is a long lasting phenomenon, with a regular curve of decay sometimes not ended after 150 msec.

The experimental analysis which suggests that the spike-like deflections of the sensory response are fiber potentials leads to the same conclusion for the spikes of the callosal response. Like the sensory spikes they are unaffected by a local strychnization or by a narcotic depression, which strongly affect the later slower components of the response. They are picked up from the underlying white matter after excision of the visual cortex. When the stimulating shock is applied directly on the relevant callosal fibers on the midline, the spacing of the spikes is reduced, a logical consequence of the shortening of the conduction distance for impulses which are supposed dispersed on account of their different velocities(12).

Conclusions. This being so, the formal analogy between the sensory and the callosal response of the visual area seems to indicate that the system of retino-geniculo-cortical fibers (and neurones) which is responsible for the characteristic pattern of the sensory response is prolonged beyond the primary visual cortex in the commissural fiber-tract connecting the left and right homologous areas. As a physiological interhemispheric transfer between homologous receiving areas (visual, auditory and somatosensory) has been demonstrated by psycho-physiological (13) and electro-physiological(14) experiments, the *raison d'être* of the callosal prolongation of this system seems to be looked for in the necessity of conserving the spatio-temporal pattern, and correlatively the information, of the sensory message during its interhemispheric transfer.

Summary. The reaction of the visual area in the unanesthetized cat to a synchronized volley of callosal impulses is markedly similar to the response of the same area produced by a shock on the optic nerve. Like this response, it is characterized by an initial succession of spike-like deflections preceding the slower

cortical potentials. Various controls (confirming Chang and Kaada's conclusion for the sensory response) indicate that these spike-like potentials are in both cases afferent fiber potentials, and thus represent presynaptic events for the cortical neurones. The significance of the formal analogy between the sensory and the callosal response of visual area is discussed. A callosal volley can exert on a subsequent sensory response of the visual area the same facilitatory effect which has been described by the author for auditory-callosal and -sensory impulses.

1. Bailey, F., and Bonin, G. von, *The Isocortex of Man,* Univ. of Illinois Press, 1951, Chp. VII, 234.
2. Nauta, W. J. H., and Backer, V. M. J., *Comp. Neurol.,* 1955, v100, 257.
3. Curtis, H. D., *J. Neurophysiol.,* 1940, v3, 407.
4. Garol, H. W., *J. Neuropathol. Exp. Neurol.,* 1942, v1, 422.
5. McCulloch, W. S., and Garol, H. W., *J. Neurophysiol.,* 1941, v4, 555.
6. Bailey, P., Garol, H. W., and McCulloch, W. S., *ibid.,* 1941, v4, 564.
7. Bartley, S. H., and Bishop, G. H., *Am. J. Physiol.,* 1933, v103, 159.
8. Chang, H. T., and Kaada, B., *J. Neurophysiol.,* 1950, v13, 305.
9. Bishop, G. H., and Clare, M., *ibid.,* 1951, v14, 497.
10. Bremer, F., *Rev. Neurol.,* 1952, v87, 162.
11. ———, *Arch. Int. Physiol.,* 1953, v51, 110.
12. Chang, H. T., *Research Public. Assn. f. Research in Nerv. a Ment. Dis.,* 1952, v30, 430.
13. Myers, R. E., and Sperry, R. W., *Anat. Rec.,* 1953, v115, 351.
14. Bremer, F., and Terzuolo, C., *J. de Physiol.,* 1955, v47, 105.

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Influence of Pyridoxine on Transaminase Activity of Human Placenta, Maternal and Fetal Blood.* (21929)

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The response of pregnant women to tryptophane(1,2) and pyridoxal loading tests(3) are characteristic of subjects with vit. B_6 deficiency(4,5). The abnormal excretion of xanthurenic acid after tryptophane loading can be abolished by daily administration of 10 mg of pyridoxine hydrochloride(6). This would imply that the altered tryptophane metabolism in normal pregnancy is the result of a deficiency in vit. B_6. Nevertheless, Vilter *et al.*(7) were "unable to demonstrate any other evidence of vit. B_6 deficiency by blood, urine, or tissue levels of this vitamin, or by measuring output of pyridoxic acid in urine." It would seem desirable, therefore, to search for some other evidence of vit. B_6 deficiency to justify large supplements of pyridoxine to diets of pregnant women. There is considerable evidence that pyridoxine derivatives, pyridoxal phosphate and pyridoxamine phosphate, serve as coenzymes of transamination reactions(8). Some tissues of pyridoxine deficient animals have a reduced transaminase activity.

Therefore, we investigated the effect of pyridoxine supplements on transaminase activity of certain fetal and maternal tissues. Blood levels were studied, inasmuch as Marsh, Greenberg and Rinehart(9) have demonstrated a correlation between pyridoxine intake and glutamic-aspartic transaminase activity of hemolyzed blood in monkeys and man. The placenta was also studied because it is the only other available fetal tissue.

Methods. Subjects were divided as follows: Group I: Ten non-pregnant healthy women who did not receive a pyridoxine supplement. Group II: Eighteen pregnant women without pyridoxine supplement.

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Group III. Eight pregnant women who received 10 mg pyridoxine hydrochloride per day during the last 4-6 weeks of pregnancy.† Transaminase activity was measured on bloods of Groups I to III and on placentas of Groups II and III. Cord bloods as well as maternal bloods were collected in 10 subjects of Group II. Blood samples from pregnant women were collected at time of delivery, using potassium oxalate as the anticoagulant. After aliquots were removed for cell counts and hematocrit levels, the remainder was repeatedly frozen and thawed in an equal volume of distilled water. Hemolyzed blood was centrifuged lightly for one minute and the clear red supernate used for transaminase determinations. Freshly delivered placentas were drained of blood, placed in plastic bags and stored in frozen state. Breis were prepared by dissection and homogenization for 20 seconds in a cold Waring Blendor. Undiluted breis were routinely freed of connective tissue and frozen before further extraction. Frozen breis retained their original activity for several months despite repeated freezing and thawing. At the time of determination, 0.5 g of freshly thawed brei were ground in a cold mortar and transferred to a graduated centrifuge tube with small portions of ice cold 0.1 M phosphate buffer, pH 7.4. The final volume was adjusted to 10 ml. The homogenate was centrifuged lightly for one minute and 0.2 ml of supernate used in each tube for transaminase determinations. Glutamic-aspartic transaminase activity was determined by a modification of the method of Tonhazy, White and Umbreit(10). The reagents were the same as those of the original method except that trichloracetic acid was omitted because of high blank values in its presence. Instead, enzyme reaction was stopped with aniline citrate to which had been added hydrochloric acid equivalent to the trichloracetic acid omitted. Blanks and standards contained the same amount of homogenate and substrates as did the reaction mixtures. The substrates (and pyruvate in the standards) were added *after* the aniline citrate reagent in the blanks and standards. Inordinately high blanks were obtained if alphaketoglutaric acid was omitted. It was necessary to use the means of triplicate determinations for blanks, standards and reaction mixtures to obtain consistent results. Some of the difficulties encountered with this method have been discussed by Marsh, Greenberg and Rinehart(9). Preliminary investigation indicated that Q_T^{10}, as defined by Tonhazy, *et al.* (10), best expressed enzyme activity for comparison between placentas. The activity of blood was expressed as the number of μl of oxalacetate formed per hour per ml of blood, calculated from the amount of pyruvate obtained after incubation of 0.1 ml of blood for 10 minutes.‡

TABLE I. Transaminase Levels in Blood.

Group	No. in group	Transaminase activity (μl oxalacetate)
I. Non-pregnant women	10	734 ± 129*
II. Pregnant women	18	748 ± 173
III. *Idem* + 10 mg pyridoxine HCl daily	6	984 ± 129
IV. Cord bloods†	10	1467 ± 194

* Means and stand. dev. of observations.
† From babies of 10 of the women of Group II.

Results. Levels of transaminase in blood samples are shown in Table I. Pregnancy appeared to have no effect. Addition of 10 mg of pyridoxine hydrochloride to the daily diet of pregnant women for several weeks resulted in a significant increase in transaminase activity ($P<0.01$). In contrast to effect on blood activity, supplements of pyridoxine had no effect on the transaminase activities of the placentas. The mean Q_T^{10} values with standard deviations were 37.9 ± 6.2 and 35.3 ± 6.0 for the supplemented and unsupplemented groups respectively. Cord bloods all contained high concentrations of transaminase activity. There was no correlation between blood levels of mothers and those of their infants. Although transaminase activity does exist in plasma(11), the method used is not sensitive enough for its measurement. Therefore, the

† We are indebted to the Stuart Co. for a generous supply of their Prenatal Capsules, some of which were specially prepared free of pyridoxine.

‡ Units, expressed as μg of sodium pyruvate/10 min./ml of blood(9), multiplied by the factor 1.22 correspond to the units used here.

TABLE II. Red and White Blood Cell Counts. Ten patients in each group.

Group	R. B. C. (millions)	W. B. C. (thousands)
I. Non-pregnant women	4.44 ± .8*	8.2 ± 1.8
II. Pregnant women (during delivery)	4.74 ±1.1	16.0 ± 3.4
IV. Cord bloods	4.76 ± .4	16.4 ± 3.7

* Means and stand. dev.

variations noted must have occurred in activities of individual cells, or in the number of cells, or both. The red cell counts of cord bloods did not differ significantly from those of any other group, nor did the white cell counts of cord bloods differ from those of mothers, although both were twice those of the non-pregnant controls (Table II). It would, therefore, appear that the activity of fetal blood cells was exceptionally high, when compared to maternal or non-pregnant subjects.

Transaminase activity of blood could be influenced very rapidly by raising the level of pyridoxine ingestion. This was demonstrated in the case of a patient hospitalized for mild toxemia (Table III). After transaminase activity had been determined on a control blood sample, 150 mg of pyridoxine hydrochloride per day were given by mouth. On the third day of supplementation, the transaminase activity showed an increase of 81%, and 32 hours later, an increase of 120%. Transaminase activity of cord blood fell within the range of those from unsupplemented mothers.

Discussion. The average American daily diet contains from 1.4 to 1.7 mg of pyridoxine (12). On the basis of observations of Marsh, Greenberg and Rinehart(9), it would be expected that a true deficiency of vit. B_6 should result in blood transaminase levels significantly lower than those found in healthy non-pregnant subjects. No significant difference was found as the result of normal pregnancy. Pregnant women responded to B_6 supplements with increased levels of blood transaminase. but this is equally true in non-pregnant subjects(9).

TABLE III. Effect of Pyridoxine Supplements on Blood Transaminase in the Same Individual.

Time of sample	Transaminase (μl oxalacetate)	R.B.C. (mill.)	W.B.C. (thous.)
8/10/54 (control level)	390	4.54	9.4
8/13/54	661	4.46	9.4
8/14 *	855	4.88	18.4
Cord blood	1660	5.10	20.8

* Immediately after delivery of baby.

Placental transaminase activity was not influenced by the pyridoxine supplements. This could mean that glutamic-aspartic transaminase activity of the human placenta, like that of the adult rat liver(13), is relatively insensitive to profound changes in vit. B_6 concentration, or more likely, that it has accumulated from maternal sources an optimal supply of pyridoxine. Transaminase activity of term placenta, expressed as units/mg of solids, is considerably higher than fetal cord blood, which itself is much higher than maternal blood.

With respect to fetal blood, the data suggest that the infant's transaminase system is well supplied with pyridoxal or pyridoxamine cofactors, perhaps at the mother's expense. If this is a "desirable situation" for the fetus. it might be reasoned that it should be desirable for the adult. The pregnant woman may be able to maintain a "normal" blood transaminase level, by non-pregnant adult standards, and still have insufficient reserve on which to draw under such conditions of stress as tryptophane loading. If one assumes, however, that increasing the transaminase level of maternal blood by pyridoxine supplements is desirable, the same reasoning would hold true for the non-pregnant adult population.

Summary. 1. The glutamic-aspartic transaminase activity of whole blood from normal pregnant subjects is essentially the same as in non-pregnant subjects. Activity is significantly increased by supplementing the diet with 10 mg of pyridoxine daily. Transaminase activity of fetal blood is twice as great as in maternal blood in mothers not receiving additional vit. B_6. The transaminase activity of placental tissue is not altered by prior administration of pyridoxine for several weeks. 2. The results suggest that fetal tissues contain optimal quantities of B_6, whereas adults, both pregnant and non-pregnant, contain suboptimal concentrations for peak enzymatic

activity. The method employed is not sufficiently sensitive to demonstrate a reduced "reserve" of B_6 in normal gestation, but the data are compatible with the view that pyridoxine supplementation is desirable in human pregnancy.

1. Sprince, H., Lowy, R. S., and Folsome, C. E., *Am. J. Obst. and Gynec.*, 1951, v62, 84.

2. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.*, 1952, v40, 1.

3. ———, *Am. J. Obst. and Gynec.*, 1953, v66, 1207.

4. Greenberg, L. D., Bohr, D. F., McGrath, H., and Rinehart, J. F., *Arch. Biochem.*, 1949, v21, 237.

5. Rabinowitz, J. C., and Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 235.

6. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.*, 1953, v42, 98.

7. Vilter, R. W., Biehl, J. P., Mueller, J. F., and Friedman, B. I., *Fed. Proc.*, 1954, v13, 776.

8. Snell, E. S., *Physiol. Rev.*, 1953, v33, 509.

9. Marsh, M. E., Greenberg, L. D., and Rinehart, J. F., *J. Nutrition*, 1955, v56, 115.

10. Tonhazy, N. E., White, N. G., and Umbreit, W. W., *Arch. Biochem.*, 1950, v28, 36.

11. Karmen, A., Wroblewski, F., and LaDue, J. S., *J. Clin. Invest.*, 1955, v34, 126.

12. Booher, L. E., and Behan, I. T., *J. Nutrition*, 1949, v39, 495.

13. Beaton, J. R., Beare, J. L., Beaton, G. H., Caldwell, E. F., Ozawa, G., and McHenry, E. W., *J. Biol. Chem.*, 1954, v207, 385.

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Effect of 2,4-Dinitrophenol on Sea-Urchin Sperm. (21930)

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2,4-Dinitrophenol (DNP) has a similar effect on many metabolic systems(1), stimulating respiration and inhibiting energy-requiring processes in low concentrations and inhibiting respiration as well as endergonic processes in high concentrations. Lardy and Phillips(2) showed that DNP stimulates respiration of bull sperm and reduces sperm motility. Since metabolism of sea-urchin sperm differs in several respects from that of mammalian sperm(3,4), it was of interest to examine the effect of DNP on sea-urchin sperm. Dinitrophenol stimulates respiratory rate and causes a reversible block to cleavage in sea-urchin eggs(5,6), but no investigations have been reported concerning the effect of DNP on sea-urchin sperm. Sperm of sea urchins and other animals exhibit "Dilution Effect" (4), characterized by rapid decrease in length of motile life and fertilizing capacity of sperm, and by temporary rise in respiratory rate, upon dilution of the suspension, the rate then falling off toward zero as sperm lose their motility. Metal-chelating agents, such as glycine and ethylenediamine tetracetic acid (Versene), can largely abolish the symptoms of the "Dilution Effect"(7-9), *i.e.* they extend the life span of sea-urchin sperm and suppress the temporary rise in respiratory rate, maintaining respiration at a low, relatively steady level.

It was, therefore, of interest to examine the effect of DNP on sea-urchin sperm in the presence of a metal-chelating agent such as Versene.

Materials and methods. Sperm from the sea urchin *Strongylocentrotus purpuratus* were used. Oxygen consumption was measured with the Warburg respirometer. Dry semen was diluted to a concentration of 4×10^8 to 6×10^8 cells per ml with sea water or with 10^{-3}M Versene† in sea water, and 3 ml of these suspensions were placed in the main compartments of 25 ml Warburg flasks; the gas space in the flasks contained air; CO_2 was absorbed with alkali. Experiments were done at 17.8°C. Dinitrophenol (0.3 ml) was added to sperm suspensions from magnetically control-

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† I am indebted to Versenes Inc. Framingham, Mass., for samples.

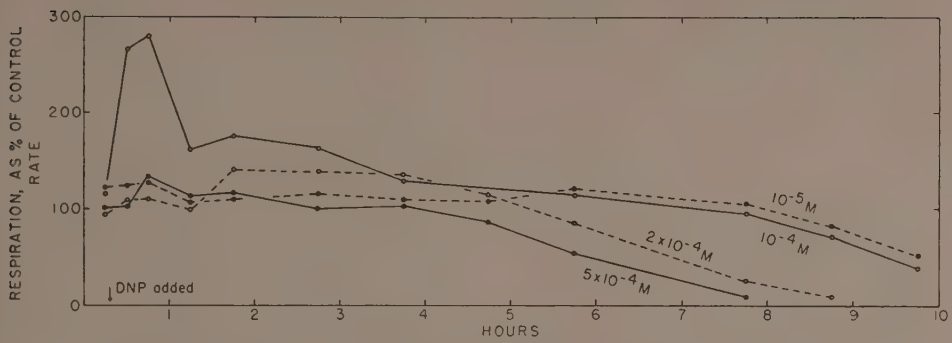

FIG. 1. Effect of DNP on *S. purpuratus* sperm suspended in 10^{-3}M Versene. Curves were determined in separate experiments; respiratory rates were averaged for duplicate flasks.

led cups to give the final concentrations mentioned below The pH of Versene and DNP solutions was adjusted to that of sea water (pH 8.00 to 8.05), and the osmotic pressures were approximately that of sea water. Triplicate test and control flasks were run in each experiment. Two flasks of each set were used for measuring oxygen consumption, and sperm motility observed periodically by removing samples from the third flask.

Results. At 10^{-4}M, 10^{-5}M, and 10^{-6}M, DNP had no appreciable effect on respiration or motility of sperm suspended in sea water. At concentrations between 2 x 10^{-4}M and 5 x 10^{-4}M, DNP inhibited respiration and motility, the degree of inhibition increasing as the concentration of DNP was increased. Inhibition of motility could be reversed by washing and resuspending the treated sperm in fresh sea water.

The response of some systems (including bull sperm) (10) to DNP depends on the initial respiratory rate of the system (1), *i.e.*, the lower the respiratory rate, the more it can be stimulated by DNP. Sea-urchin sperm have a relatively high respiratory rate when suspended in sea water because of the "Dilution Effect." Thus, it seemed possible that if the respiratory symptom of the "Dilution Effect" were suppressed, DNP might then stimulate sperm respiration. Respiration was controlled by suspending the sperm in 10^{-3}M Versene (9), the sperm in this diluent having an initial respiratory rate from 25% to 65% of the initial rate of sperm in sea water.

Sperm in Versene were treated with 10^{-5}M, 10^{-4}M, 2 x 10^{-4}M, and 5 x 10^{-4}M DNP. At 10^{-4}M, DNP initially stimulated respiration to 280% of the control rate (Fig. 1), the respiratory rate of the treated sperm falling below the control rate within 8 hours. The other concentrations of DNP gave less stimulation, and respiration fell below the control rate more quickly as concentration of DNP was increased. Except at 5 x 10^{-4}M, DNP did not detectably inhibit sperm motility until after the respiratory rate of treated sperm fell below that of controls. At 5 x 10^{-4}M, DNP inhibited the motility of Versene-treated sperm by about 40% (visual estimation) within 20 minutes, and the life span of treated sperm was shorter than that of control sperm (Fig. 2).

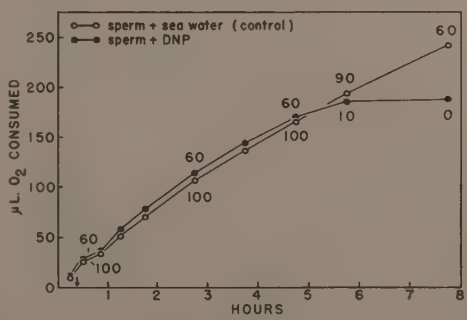

FIG. 2. Inhibition of motility and dissociation of respiration from motility by 5 × 10^{-4}M DNP, sperm in Versene (10^{-3}M). Points represent averages for duplicate flasks. Numerals represent motility ratings (visual estimation), initial motility rating = 100. Arrow indicates when DNP was added to sperm. (After curves cross, motility ratings are still placed next to curve to which they refer.)

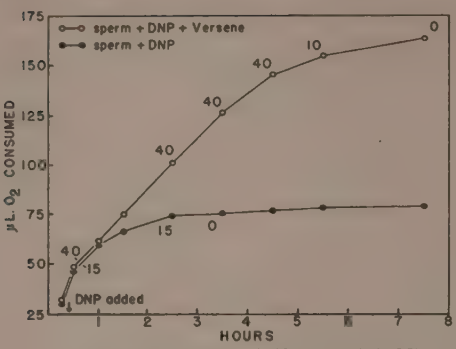

FIG. 3. Protective effect of Versene (10^{-3}M) on *S. purpuratus* sperm in 5×10^{-4}M DNP. Points represent averages for duplicate flasks. Numerals represent motility ratings (visual estimation), initial motility rating = 95.

Versene, as compared to sea water, had a protective effect on sperm treated with 5 x 10^{-4}M DNP (Fig. 3). When DNP was added to sperm in sea water motility was inhibited within 15 minutes, and the sperm rendered completely immotile within 3 hours. Versene, added simultaneously with DNP, reduced the initial inhibition of motility and prolonged the life span and respiration of DNP-treated sperm for 3 to 5 hours.

Unbuffered sea water was used in these experiments, because buffers that might have been used, such as glycylglycine(11), would be likely to affect respiration by virtue of chelating or other specific effects. As a result, the pH of the sperm suspensions in the flasks increased with time (as sperm respiration declines and less respiratory CO_2 is produced, the KOH in the alkali cup begins to extract CO_2 from the sea water diluent). Since DNP penetrates cells more easily as an undissociated molecule than as an ion(12), pH changes alter the effective concentration of the inhibitor. In these experiments, however, the pH of the sperm suspensions in the Warburg flasks did not change appreciably for 1 to 3 hours, indicating that the differential effect of DNP in the presence and absence of Versene was not due to differences in pH of the suspending media.

Discussion. Dinitrophenol at low concentrations can inhibit energy-requiring processes by blocking oxidative phosphorylation and thereby reducing the amount of adenosine-triphosphate available to the cell(13). Since respiration is stimulated as this inhibition occurs, DNP, in effect, "uncouples" respiration from endergonic metabolism. Dinitrophenol apparently effects some "uncoupling" of respiration and motility with *S. purpuratus* sperm. Thus, at 5 x 10^{-4}M DNP inhibits motility while slightly stimulating respiration (Fig. 2). At lower concentrations, however, DNP does not inhibit motility as it stimulates respiration, but "uncoupling" occurs in the sense that the increase in respiratory rate is not accompanied by an increase in sperm activity.

In addition to inhibiting oxidative phosphorylation, DNP also stimulates the adenosine triphosphatase (ATPase) activity of some tissues(14), the two effects sometimes being interrelated(13). It is interesting, then, that Versene and DNP have the same qualitative effect on the activity of rat-brain ATPase(15) as they have on the respiration of sea-urchin sperm. Thus, DNP stimulates the ATPase activity of rat-brain homogenates, but only after the initially high activity of the preparations has been reduced by treatment with ethylenediamine tetracetate.

The effect of DNP on respiration and motility of sea-urchin sperm may involve both an inhibition of oxidative phosphorylation and a stimulation of sperm ATPases.

It is apparent that DNP and dilution have similar effects on *S. purpuratus* sperm. Both shorten the life span and stimulate respiration of sperm, DNP not having a stimulatory effect until that of dilution has been abolished by Versene. Also, both seemingly cause some dissociation of respiration and motility; for dilution this is suggested by the fact that Versene reduces the high respiratory rate of diluted sperm without reducing sperm motility.

As the concentration of DNP is increased, DNP and the agent responsible for the "Dilution Effect" seem to have an accumulative inhibitory effect on sperm respiration and motility.

These similarities raise the possibility that the effects of dilution and DNP occur through similar mechanisms. Thus the "Dilution Effect" may involve an inhibition of oxidative phosphorylation or an increase in the activity

of a sperm ATPase, perhaps one not associated with motility.

Summary. Low concentrations of DNP did not stimulate respiration or affect motility of sea-urchin sperm suspended in sea water; high concentrations of DNP inhibited sperm respiration and motility, the inhibition of motility being reversible. Dinitrophenol stimulated the respiratory rate of sperm in the presence of Versene and caused respiration to be partially dissociated from motility. Versene, as compared with sea water, prolonged the motile life and respiration of sperm treated with high concentrations of DNP. Dinitrophenol and dilution have similar effects on sea-urchin sperm, raising the possibility that both act through similar mechanisms.

1. Simon, E. W., *Biol. Rev.*, 1953, v28, 453.
2. Lardy, H. A., and Phillips, P. H., *J. Biol. Chem*, 1943, v149, 177.
3. Mann, T., *The Biochemistry of Semen*, Methuen and Co., London, 1954.
4. Rothschild, Lord, *Biol. Rev.*, 1951, v26, 1.
5. Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1936, v20, 145.
6. Tyler, A., and Horowitz, N. H., *Biol. Bull.*, 1938, v75, 209.
7. Tyler, A., and Rothschild, Lord, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 52.
8. Tyler, A., *Biol. Bull.*, 1953, v104, 224.
9. Rothschild, Lord, and Tyler, A., *J. Exp. Biol.*, 1954, v31, 252.
10. Melrose, D. R., and Terner, C., *Biochem. J.*, 1953, v53, 296.
11. Tyler, A., and Horowitz, N. H., *Science*, 1932, v86, 85.
12. Tyler, A., *Proc. Nat. Acad. Sci.*, 1937, v23, 369.
13. Hunter, F. E., Chap. 5 in *Symposium on Phosphorus Metabolism*, v1, Johns Hopkins Press, Baltimore, 1951.
14. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, 1953, v201, 357.
15. Maxwell, R. E., and Nickel, V. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 846.

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Effect of Age on Polysaccharide Composition of Cartilage. (21931)

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Human cartilage has been subjected to chemical analysis by Hass(1) who studied the effect of aging on its composition. His methods for studies of polysaccharide components consisted of the determination of reducing substances and of sulfate in washed cartilage after hydrolysis with 4.2 N HCl. All of the sulfate and one-half of the reducing substances were assumed to be derived from chondroitin-sulfuric acid under these circumstances. As methods for hexosamine, hexose, and uronic acids are now available and have been used in this laboratory for studies of tissue, it is possible to make more specific studies of the polysaccharide moieties of cartilage. The following study was accordingly initiated.

Methods. Costal cartilage samples were obtained at autopsy from patients who had died from a number of different pathological conditions. The cartilage was freed from extraneous tissue, cut in fine pieces with a surgical knife and dried in a vacuum oven at 60°C. The dried cartilage was ground to pass through a 60 mesh screen.

Uronic acid was determined by a method which was modified from the carbazole method of Dische(2). Ten mg samples of cartilage were weighed into glass stoppered test tubes (16 x 150 mm). Ten ml of 85.7% by volume of sulfuric acid (concentrated H_2SO_4; Sp. Gr. 1.84, diluted 6:1 with distilled water) were added and the tubes placed in a boiling water bath for 15 minutes. After cooling 2 ml aliquots were pipetted into each of 3 glass stoppered test tubes. Twelve ml of 85.7% sul-

TABLE I. Uronic Acid and Hexosamine Contents* of Human Costal Cartilage.

Age group	No.	Uronic acid	Chondroitin sulfate†	Hexosamine Total	Hexosamine Excess‡
Fetal	5	7.8 (7.4–8.0)	19.1	8.2 (7.9–8.5)	1.0 (.6–1.1)
Newborn	2	7.5 (7.3–7.7)	18.4	8.7 (8.5–8.9)	1.3 (1.2–1.4)
4 mo	1	7.0	17.2	7.1	1.6
6–11 yr	2	3.6 (3.6–3.7)	9.0	6.1 (6.0–6.2)	2.7 (2.6–2.8)
25–40 "	5	3.3 (2.9–3.9)	8.0	7.3 (6.9–7.7)	4.3 (3.4–4.6)
41–55 "	5	2.4 (2.0–3.2)	5.8	6.8 (5.4–7.7)	4.6 (3.9–5.1)
56–70 "	10	1.6 (1.3–1.9)	4.0	6.1 (4.9–6.6)	4.6 (3.6–5.0)
71–88 "	4	1.3 (1.1–1.6)	3.2	5.4 (4.7–6.0)	4.3 (3.6–4.6)

* All results are expressed as % of dry material.
† Calculated from uronic acid data using factor 2.46.
‡ Hexosamine in excess of that calculated to be in the chondroitin sulfate.

furic acid were added to each tube. The tubes were cooled 10 minutes in an ice bath. 0.4 ml of carbazole reagent (0.1 g/100 ml absolute ethanol) was added to 2 of the tubes; 0.4 ml of absolute ethanol was added to the third tube which served as an experimental blank. The tubes were mixed by inversion and placed in a 37°C incubator for 1 hour and then in a 30°C water bath for 1 hour. Samples were then read in a Coleman Spectrophotometer at 530 mu against the respective blank. A standard solution of glucuronic lactone* equivalent to 100 μg of glucuronic acid in 2 ml of water was cooled 10 minutes in an ice bath and then diluted with 12 ml of concentrated H_2SO_4. It was hydrolysed for 20 minutes in the boiling water bath. After cooling for 10 minutes in the ice bath, the carbazole reagent was added and the tubes were carried through the procedure as described above. This standard was read against a blank containing glucuronic lactone, sulfuric acid and absolute alcohol, subjected to the same procedure. Hexosamine was determined by a modified Elson-Morgan method as previously described by Shetlar, *et al.*(3).

Results. Results of analytical determinations are summarized in Table I. It can readily be seen that uronic acid is quite high in young cartilage, but decreases drastically in cartilage of children and mature individuals and decreases even more in old age. Hexosamine content also decreases with age, but the decrease is much less striking than it is for uronic acid. If one assumes that all the uronic acid of cartilage is contained in chondroitin sulfuric acid, the latter component may be calculated from the uronic acid content. The excess hexosamine may logically be assumed to be contained in neutral polysaccharides with hexosamine and hexose as carbohydrate constituents. These calculated data indicate that although the acid polysaccharide of cartilage decreases with age, neutral polysaccharide increases in cartilage until maturity. After maturity no appreciable change of neutral polysaccharide occurs.

Discussion. The chondroitin sulfate values reported in Table I are in general somewhat lower than those reported by Hass(1). These differences may be due to the different methods used to arrive at the values found for chondroitin sulfate. Hass assumed that one-half the reducing substance and all of the sulfate of cartilage was derived from the chondroitin sulfate. Obviously these are only approximations. It is noteworthy that results obtained by isolation as reported by Hass are much lower. cartilage from 35, 46, 50, and 66-year-old individuals having 11.4, 8.5, 6.9, and 5.8% of chondroitin sulfate, respectively. These results compare more favorably with the data of Table I. The other possibility is that the uronic acid determinations made as described above are too low. In the presence of some proteins the carbazole method does give results which are too low(2). However, optical densities of glucuronic acid lactone solutions carried through the analytical procedure described above were not affected by the presence of gelatin in concentrations equivalent in nitrogen content to that found in cartilage. Further studies of the carbazole method are in progress.

* General Biochemicals, Chagrin Falls, O.

In any case the data of Table I indicate chondroitin sulfate is higher in young cartilage and decreases with age. Hass reported an increase between ages 2 and 21 followed by a gradual decline. The data obtained in this study confirmed the gradual decline after 21 years, but were insufficient between the ages of 2 and 21 to confirm or fail to confirm the observation of an elevation between these ages. Hass' studies did not include cartilage samples from fetal or of newborns which have a uronic acid content much higher than that of children. When such data are considered, it appears that a high acid polysaccharide level is characteristic of young growing cartilage and may be correlated with active proliferation. A more rapid fixation of sulfate in young tissue has been reported(4,5). This fixation in part is probably a reflection of a higher chondroitin sulfate content.

Conclusions. A study was made of the polysaccharide constituents of cartilage from individuals of various ages. Fetal cartilage was found to be very high in uronic acid. The uronic acid content was strikingly lower by the sixth year and then declined slowly with age. Hexosamine in excess of uronic acid increased with age until maturity was reached after which there was no appreciable change.

The authors are indebted to Dr. C. D. Tool, Pathologist, VA Hospital, for samples of adult cartilage and to Miss Jeanne Green, Pathology Department, University of Oklahoma School of Medicine for samples of fetal cartilage.

1. Hass, George M., *Arch. Path.*, 1943, v35, 275.
2. Dische, S., *J. Biol. Chem.*, 1947, v167, 189.
3. Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 507.
4. Layton, L. L., *Cancer*, 1950, v3, 725.
5. Layton, L. L., Denko, C. W., Scapa, S., and Frankel, D. R., *ibid.*, 1952, v5, 405.

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Complement-Fixing Antigens in Concentrates of Streptococcal Culture Supernates.* (21932)

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Recent research on the etiology of rheumatic fever has been directed at the only known association of this disease with any microorganism—association with hemolytic streptococcus. This relationship, which has been reviewed in terms of epidemiologic(1), serologic(2,3) and bacteriologic(4) data, suggested to several investigators that some immunologic relationship might exist between a substance derived from the hemolytic streptococcus and the tissues of the rheumatic host. Within the area of metabolic products of the streptococcal cell there has been considerable study of those antigens which have some biologic or biochemical activity, and to which antibodies can be measured in neutralization tests. Such streptococcal antigens include the hemolysins, hyaluronidase, desoxyribonuclease and kinase (plasminogen activator). Other possible antigens, to which antibodies would be measurable by aggregation phenomena, have not been studied except for 2 studies of hemagglutination reactions with streptococcal supernate concentrates(5,6). The present report concerns an investigation of this potential group of streptococcal antigens.

Methods and materials. Medium. Streptococci were cultivated in either of 2 media, a dialysate medium and, for the most part, in the synthetic medium described by Bernheimer and Pappenheimer(7). The dialysate

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medium was prepared by dissolving dehydrated dextrose broth (Difco) at 20 times the concentration used in preparing ordinary medium and dialyzing this solution in cellophane bags against 19 volumes of distilled water in a rotating dialyzer for 18 hours at 4°C. The pH of the resulting dialysate, (6.5), was brought to 7.2 by addition of molar NaOH. After sterilization in the autoclave, one-tenth volume of sodium bicarbonate was added. This medium, or the synthetic medium, was seeded with an overnight culture of β-hemolytic streptococci, the inoculum being 4% of the volume of the medium. *Concentration of culture supernates.* The combined cultures of each day were pooled, after adjustment of the pH to approximately its original value, distributed into cellophane bags hung on a rack before an electric fan for concentration by pervaporation. After 16 hours of pervaporation at about 12°C, the contents of the bags, now concentrated approximately 10-fold, were pooled and subjected to vigorous centrifugation (30 minutes at 15,000 r. p. m.) to remove bacterial cells. This concentrate was dialyzed overnight against running tap water and then against distilled water, after which the volume was further reduced by pervaporation for 6 hours at 12°C. The pooled concentrates were then desiccated from the frozen state, and the dried material was stored at 4°C. Similar preparations could be obtained by ammonium sulfate precipitation of Seitz filtrates of the cultures but this method produced unsatisfactory yields because of loss of antigenic material by adsorption on the asbestos pad. The strains of β-hemolytic streptococci of Lancefield Group A, were obtained through the courtesy of Dr. Rebecca C. Lancefield. They were maintained in lyophilized form for use in seeding cultures as described above. The human sera obtained from presumably healthy adults in the area of Philadelphia were outdated sera supplied through the courtesy of the Philadelphia Serum Exchange.

Technic of the complement-fixation test. The complement-fixation test was the serologic test used in almost all the work described. When used for estimation of antigenic material, the solution of the latter was prepared in various dilutions, usually in 2-fold steps of dilution, in 0.4-cc volumes. These dilutions were incubated 45 minutes at 37°C with 0.1 cc of a standard serum, diluted to its optimal concentration for this test, and 0.1 cc of guinea pig serum, diluted to contain 1.3 units of complement in this volume. After incubation each tube received 0.1 cc of 4% sheep erythrocytes (adjusted so that 1 cc of the suspension, hemolyzed and diluted to a final volume of 20 cc, yielded a reading of 135 in the Klett photoelectric colorimeter with a #54 filter), and 0.1 cc of rabbit anti-sheep-erythrocyte serum diluted to an optimum concentration for this hemolytic system. For the measurement of antibody the same system was employed, with serial dilutions of serum, and a constant amount of antigen preparation.

Results. A. Production of complement-fixing antigen(s). 1. Observation of complement-fixing antigens in concentrates of streptococcal culture filtrates. Concentrates of the supernates of β-hemolytic streptococcal cultures (Group A, type 4, strain H44) in both dialysate and synthetic media were tested by complement fixation in block titration against sera of rabbits, given long courses of injections of killed, and then living streptococcal cells. These rabbits had received streptococci of type 1. Typical results of such complement fixation tests are shown in Table I. It can be seen that a complement fixation reaction is demonstrable between concentrates of streptococcal culture filtrates and rabbit antistreptococcal serum. Antigenic material of streptococcal origin was designated CSA (culture supernate antigens). In exploratory work with these preparations, a small percentage of sera obtained from healthy adults contained complement-fixing antibodies to the streptococcal CSA in a range of titers from those barely measurable by the technic described here up to 32. The relatively higher-titered sera among these were used as reagents for the measurement of CSA, reported below. Table I also shows the results of a complement-fixation test of the same preparations of streptococcal CSA against such a human serum.

Uninoculated synthetic medium was concentrated as described above. The yield of

TABLE I. Complement-Fixation Tests of Streptococcal CSA Produced on Broth-Dialysate Medium and on Synthetic Medium Versus Rabbit Antiserum and Human Serum.

	CS2E (dialysate medium)					CS26 (synthetic medium)					
	Concentration, γ/cc					Concentration, γ/cc					
	250	125	63	31	16	31	16	8	4	2	0
Human serum C											
Diluted: —	0	0	0	1+	2+	0	0	0	2+	2+	2+
1:2	0	0	0	1+	3+	0	0	tr	2+	3+	3+
1:4	0	0	2+	3+	3+	0	2+	3+	3+	3+	3+
1:8	1+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Rabbit antiserum 232											
Diluted: 1:8	0	0	0	2+	3+	0	0	tr	2+	3+	3+
1:16	0	0	0	2+	3+	0	0	0	1+	3+	3+
1:32	0	0	1+	3+	3+	0	0	2+	3+	3+	3+
1:64	0	1+	3+	3+	3+	0	3+	3+	3+	3+	3+
—	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

Degrees of hemolysis: 0, none; 1+, weak; 2+, strong; 3+, complete.

dry weight was 0.4 mg per liter, of the order of one-fortieth the yield of dry weight of CSA per liter of culture. Complement-fixation tests as described above were entirely negative, when this material was used in the same order of concentration as shown for CSA in Table I.

2. *Production of CSA by various types of streptococci of Group A.* To obtain data as to breadth of reactivity of the antigenic material and extent of its production by various types, 6-liter cultures were grown of each type, of a collection of 36 types available. The filtrate of each culture was concentrated, lyophilized and tested for its content of CSA by complement fixation against the rabbit serum mentioned above. In the available strain of each of 5 types, 6, 12, 13, 23, 37, no CSA was found within the threshold of sensitivity of our measurements. In concentrates derived from the other 31 types, CSA was found in a range of concentration from 1 complement-fixing unit per mg of lyophilized material to 16, in the case of our strain of types 25, 30, 36, and 40.

3. *Production of CSA in relation to growth of organisms.* The relationship of concentration of CSA to growth of streptococci was studied as follows: Cultures of several types were seeded into dialysate medium, and at various intervals (0, 4, 8, 12, 24 hours) samples of the well mixed cultures were withdrawn into tubes for immediate centrifugation. The sediment was washed once with saline solution by centrifugation and resuspended in the original volume of the sample for a measurement of turbidity (Klett densitometer, #54 filter). The supernate was dialyzed overnight against running tap water in a ¼ inch cellophane tube and then concentrated by pervaporation to half the original volume. This concentrated culture filtrate was used for the measurement of CSA by complement fixation. In some cultures it was found that the greatest part of the rise in concentration of CSA occurred before the 8th hour of culture, at which time CSA concentration was 1.5 units/cc, and the turbidity of the resuspended bacterial sediment 65; 4 hours later the CSA concentration had risen to 1½ times their value at 8 hours, and the turbidity of the bacterial sediment to 152. Thereafter there was no substantial increase in the value of either quantity. In other cultures it was found that up to 12 hours the increase in the CSA concentration was in general parallel to the increase in the turbidity of the resuspended bacterial sediment, but thereafter the latter increased without corresponding increase in CSA concentration.

B. *Characterization of CSA.* 1. *Effects of pH.* Experiments to determine the effects of high and low pH on activity and solubility of CSA were carried out on solutions of lyophilized concentrates derived from cultures in the synthetic medium. Samples of such solutions were brought to given values of pH

TABLE II. Effect of Previous Incubation with Erythrocytes on Hemolytic and Complement-Fixing Activity of a Preparation of CSA.

	Incubation mixture, 5 ml of 0.4% CSA 82, incubated with														
	A					B					C				
	.6 ml PSS (control)					.6 ml packed RBC and 10 mg $NaHSO_3$					.6 ml packed RBC				
	Tested at dilution of														
	1	2	4	8	16	1	2	4	8	16	1	2	4	8	16
Complete fixation vs. rabbit antiserum No. 1685 at dilutions:															
64	0	0	0	tr	2+	0	0	0	1+	2+	0	0	0	1+	2+
128	0	0	0	tr	2+	0	0	0	1+	2+	0	0	0	1+	2+
256	1+	1+	1+	1+	3+	1+	2+	2+	2+	3+	1+	2+	2+	3+	4+
Hemolytic activity vs. 1 vol. of 0.4% RBC	4+	4+	4+	4+	1+	0	0	0	0	0	4+	4+	4+	1+	1+*

* $NaHSO_3$ added to this preparation for the hemolysin test.
Degrees of hemolysis: 0, none; 1+, weak; 2+, strong; 3+, almost complete; 4+, complete.

by addition of normal hydrochloric acid or ammonium hydroxide, the reagents being added dropwise to the chilled solution with constant stirring. The solutions were kept overnight at 4°C, at the desired pH, after which any precipitate which had formed was removed by centrifugation. Sediments were dissolved in a volume of buffered saline solution equal to that of the original volume, and the pH of the supernate was restored to its original value. Each fraction was then tested for CSA activity by complement fixation, in comparison with the original solution. Between pH 5 and 8.5 there was no precipitation and no loss of activity. At pH 4 a flocculent precipitate was obtained, and the activity of CSA was divided approximately equally between sediment and supernate. At pH 3.5 a more bulky precipitate was obtained, and all of the original activity could be found in the redissolved sediment, no antigen being detectable in the supernate. At pH 1 there was no activity in the supernate, and only half activity could be recovered from the redissolved sediment. At pH 9 a precipitate was obtained, but all serologic activity of the original material could be found in the supernate. At pH 10 a larger, inactive, precipitate was obtained and some loss of activity of the supernate was found. The extent of precipitation of inactive material at pH 9 was not sufficient to increase measurably the activity per mg of the preparation under present conditions of testing.

2. *Precipitation by ammonium sulfate.* A 4% solution of CSA was gradually brought to 50% of saturation with ammonium sulfate by equilibrating the solution with one volume of a saturated neutralized solution of the salt. Additional solid ammonium sulfate was added to saturation. The resulting suspension was neutralized and refrigerated overnight. The sediment was collected by centrifugation on the following day, washed with a saturated solution of ammonium sulfate, and dissolved in distilled water. This solution was dialyzed against running water overnight, in cellophane tubing of ¼″ diameter. The supernate was similarly dialyzed. The volume of the redissolved sediment was then brought to the original volume of the solution of CSA, and the redissolved sediment and supernate were tested for complement-fixing activity. The activity of the original solution was completely recovered in the ammonium sulfate sediment, and no activity (less than 3%) appeared in the supernate. Analyses for nitrogen in the case of 3 pools of the CSA as produced in the synthetic medium yielded a mean value of 13.6% N relative to dry weight.

3. *Relation of complement-fixing antigens with other streptococcal antigens.* a. *Hemolysin-O.* The question of the relationship of streptococcal hemolysin-O to the complement-fixing antigens in CSA was approached by using erythrocytes as adsorbents of hemolysin. A solution of CSA, which contained both complement-fixing and hemolytic

TABLE III. Comparison of Complement-Fixation Titers of Various Preparations of Streptococcal Culture Supernates vs. Rabbit Antiserum with Activities of 3 Enzymes in Those Preparations. All values refer to relative concentrations of respective reagents in solutions of culture supernate concentrates at 1 mg/cc.

Preparation	Strain	CF titer	Hyaluronidase titer	Streptokinase titer	Desoxyribonuclease titer	Ratios CF:H	Ratios CF:S	Ratios D:CF
130B	5797	200	<4	16	2000	>50	12.5	10
100A	H105	24	6	24	800	4	1	33
H7	NY5	1	4	<1	64	.25	>1	64
134B	H44	400	4	64	8000	100	6.3	50
H pool	H44	.5	16	<1	96	.03	> .5	192
CS "	H44	400	256	500	6000	1.6	.8	15
C3	H44*	12	96	<1	6000	.13	>12	500
CS 217	RG†	48	1	<1	3000	48	>48	63
CS 224 A	RJ†	32	.5	<1	1500	64	>32	47
CS 225	EC†	24	1	<1	1500	24	>24	63
—	—	—	—	—	—			
				Range of ratios:		>1600-fold	>60-fold	50-fold

* Cultivated in the Chemostat, under constant chemical conditions.
† Fresh human isolation, from non-inflamed pharynx.

activity, was incubated with a suspension of sheep erythrocytes at 4°C, both reagents having been chilled before incubation. After 30 minutes of such incubation erythrocytes were removed by centrifugation in a refrigerated centrifuge, and the supernatant fluid was tested for complement-fixing and hemolytic activity. The results are shown in Table II. It can be seen, on comparison of parts A and B, that the concentration of hemolysin in this preparation has been reduced to less than 6% by the incubation with erythrocytes, whereas the complement-fixing activity of this preparation has been reduced very slightly, if at all. Comparison of the data in part C with that in part B indicates that adsorption of hemolysin to erythrocytes takes place only if the former is in the reduced form. The fact that the very slight loss of complement-fixing activity in preparation B is also found in C would suggest that this loss was not related to the adsorption of hemolysin.

b. *Hyaluronidase, desoxyribonuclease and streptokinase.* Preparations of CSA derived from various cultures were tested for hyaluronidase, desoxyribonuclease and streptokinase activity in simultaneous tests, to obtain data on the association of any of these enzymes with the complement-fixing material. The results of some of these tests are shown in Table III. For inclusion in this Table, preparations were chosen which showed a relatively wide range of ratios of measures of relative activity between complement-fixation and the respective enzymes. Table III shows that the range of ratios of complement-fixation titers to hyaluronidase titers was from 0.03 to >50, and that of streptokinase to complement fixing titers was from 0.8 to >48. The range of ratios of desoxyribonuclease to complement-fixation values was from 10 to 500.

c. *Complement-fixing somatic streptococcal antigens.* In earlier studies the preparation and complement-fixation reactions were described of 2 antigens or groups of antigens derived from extracts of sonically-disrupted streptococcal cells. These were designated as CP (cytoplasmic particles) and S (supernate proteins)(8,9). To obtain data on possible relationships between complement-fixing materials derived in the present study from the culture supernate, and those obtained by disruption of the streptococcal cell, a number of human sera were included in simultaneous complement-fixation tests against all 3 antigenic preparations, each used at its optimal concentration. The results of some of these tests are shown in Table IV. Data are presented in the case of 10 sera which include a relatively wide range of ratios of antibody titers to the 3 antigenic preparations. The range of ratios of anti-CSA to anti-CP titers in respective sera was from less than 0.1 to 2, and the range of anti-CSA titers to anti-S titers was from 0.5 to greater than 16.

Discussion. 1. *Production of complement-*

TABLE IV. Complement-Fixation Titers of 10 Sera Tested with 3 Streptococcal Preparations, CSA and 2 Somatic Fractions, to Illustrate Ranges of Relative Titers.

Serum No.	Titers vs.: CS	CP	S	Ratio of: CS/CP	CS/S
103	96	512	128	.2	.7
3821	12	128	8	.1	1.3
3955	128	96	12	1.3	11
4479	16	256	32	.07	.5
5119	128	192	8	.7	16
5267	128	128	12	1	11
5308	32	96	32	.3	1
5417	64	64	8	1	8
5657	768	384	128	2	6
5717	8	128	8	.07	1

fixing antigens in streptococcal culture. The data presented above would indicate that the substances which give rise to complement fixation with the sera used, are produced by the streptococcal cells in culture. This is suggested by the low yield in dry weight of uninoculated medium concentrated by similar procedures, by the negative complement-fixation tests found with such concentrates of the uninoculated medium, and by the concomitant rise in concentration of complement-fixing antigens in culture supernate with the turbidity of the culture. This antigenic material is broadly reactive, at least within Group A of β-hemolytic streptococci, since cultures of strains of almost all types within this group produced varying amounts of it. That this material, or at least a large part of it, is protein, is suggested by the fact that it is completely precipitable by ammonium sulfate at 80% of saturation, and by the mean nitrogen value of 13.6%. 2. *Relation of streptococcal CSA to known streptococcal antigens.* The question of identity of this complement-fixing material or of any components of it with any of the known streptococcal antigens cannot be properly approached until separation of such components has been achieved. In the single case of the O-hemolysin it has been possible to adduce fairly direct evidence that this substance is not responsible for the fixation of complement found with CSA, or at least for a major portion of it, since the adsorption of hemolysin from the crude concentrate by erythrocytes was not accompanied by a decrease in complement-fixing activity. The data on relationship of complement-fixing substances in CSA with other known streptococcal antigens is quite indirect and can be considered only as suggestive. Comparison was made of the concentration of complement-fixing antibodies to CSA with those to somatic nucleoprotein fractions of streptococcal cells to obtain some evidence as to whether the CSA might be largely derived from such cell fractions by dissolution of bacterial cells in culture. The wide range of ratios of antibody titers shown in Table IV would suggest that the fixation of complement by any cellular nucleoprotein which might have been released into the culture medium, could not have accounted for a major portion of the complement fixation in the tests with CSA.

The other group of antigens, examined in part, is that of the extracellular enzymes which, like the CSA, are found in the culture supernatant, and which might as antigenic proteins fix complement with homologous antibodies. While a complete list of such antigens was not examined, in experiments such as those reported in Table III, the wide disparity among measurements of complement-fixing activity, on one hand, and any of the 3 enzymatic activities, on the other, makes it quite unlikely that any of these 3 enzymes are responsible for a major portion of the complement fixation observed. Although there are, of course, other known streptococcal extracellular enzyme-antigens, the number of immunologically distinct antigenic species which would appear to be present in streptococcal CSA according to the data presented in the following paper (at least 7) would render it quite unlikely that the other streptococcal extracellular enzymes could be identical with the respective antigens found in this material. It would appear highly likely, therefore, that an antigen or antigens of streptococcal origin, not identical with any of the known streptococcal antigens, are present in the concentrates of streptococcal culture supernates.

Summary. 1. Supernates of cultures of β-hemolytic streptococci were concentrated with respect to macromolecular material present. Such concentrates gave positive complement-fixation reactions with sera of rabbits which had been given courses of injections of living

streptococci, and with sera of human subjects. 2. Evidence was obtained that complement-fixing antigenic material in such preparations was not identical with the complement-fixing fractions obtained from streptococcal cells, and complement fixation was probably not due to any of a number of the extracellular enzyme-antigens which can be found in streptococcal culture supernates.

1. Paul, J. R., and other contributors, *The epidemiology of rheumatic fever and some of its public health aspects*, ed. 2, New York, Metropolitan Life Insurance Company, 1943.

2. Harris, T. N., *Am. J. Dis. Child.*, 1948, v76, 411.

3. McCarty, M., in *Rheumatic Fever, a symposium*, Thomas, L., ed., p136, University of Minnesota Press, Minneapolis, Minn., 1952.

4. Green, C. A., *J. Path. and Bact.*, 1941, v53, 223.

5. Kirby, W. M. M., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 519.

6. Harris, T. N., and Harris, S., *J. Bacteriol.*, 1953, v66, 159.

7. Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., Jr., *ibid.*, 1942, v43, 405.

8. Harris, T. N., *J. Exp. Med.*, 1948, v87, 41.

9. ———, *ibid.*, 1948, v87, 57.

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Gel-Precipitation of Streptococcal Culture Supernates with Sera of Patients With Rheumatic Fever and Streptococcal Infection.* (21933)

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In the literature dealing with antigenic substances produced by the hemolytic streptococcus in liquid cultures the emphasis has been almost exclusively on those antigens which have some biologic activity, such as toxicity or enzymatic function. In present studies the reactions of tissues of the rheumatic host to streptococcal antigens are explored. These studies are concerned not only with streptococcal antigens which have known enzymatic function, but also with those which are demonstrable only by their ability to give rise to antibody formation and to combine with those antibodies in mass reactions such as complement-fixation or precipitation. Production and concentration of macromolecular materials produced in streptococcal culture, and their reaction in complement-fixation tests with sera of injected rabbits and human subjects are described in the preceding paper (1). The complement fixation test does not, however, distinguish between one or more immunologic systems in solution (except in quite special circumstances of wide difference of concentrations of reagents relative to their specific activity). For the purposes of these studies methods of greater resolution were necessary to examine the antigenic materials produced in streptococcal culture. Accordingly, such materials were studied by gel-precipitin tests against rabbit and human sera. During such studies differences were noted in the numbers of antibodies present in sera of various groups of human subjects, in sufficient concentration to be observed. These will be presented here.

Methods and materials. 1. *Antigenic material.* Streptococci of Lancefield group A (Type 4, Strain H_{44}) were cultivated for 18 hours in the synthetic medium described by Bernheimer *et al.*(2). Supernates of centrifugation of such cultures were concentrated by precipitation with ammonium sulfate (at 80% saturation) in the presence of a cellulose fiber filter aid, Solka Floc. The extract of the resulting sediment was collected, suitably

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dialyzed and desiccated from the frozen state. The dry material was weighed and solutions of this material were used as described below. This material will be referred to as streptococcal CSA (culture supernate antigens).

2. *Sera.* Rabbits were given injections of CSA in either of two forms: weekly injections of approximately 5 mg CSA precipitated with alum, or tri-weekly injections, each 2.5 mg of the material in solution. Sera were obtained after a 4-week initial course of such injections and then, after successive 2-week courses of such injections, separated by one-week intervals. Human sera were obtained from 3 sources. Sera of presumably normal human subjects were obtained from outdated refrigerated bloods through the courtesy of the Philadelphia Serum Exchange. Sera of patients with scarlet fever were obtained from patients with this disease at the Philadelphia General Hospital at the beginning and end of their 3-week period of hospitalization, through the courtesy of Dr. A. C. LaBoccetta. A later specimen was obtained from a number of these patients approximately 6 weeks after the onset of streptococcal infection. Sera of patients with rheumatic fever were obtained from patients with a definite diagnosis of active rheumatic infection who were studied in the wards of the Philadelphia General Hospital, the Hospital of the University of Pennsylvania and the Children's Hospital of Philadelphia. Sera were obtained at the beginning of hospitalization and at 2-month intervals thereafter. Sera of patients with inactive rheumatic heart disease were obtained from patients at the Rheumatic Fever Clinic of the Philadelphia General Hospital. These patients were known to have had rheumatic fever at some earlier time (at least 2 years earlier) from study in the wards of above hospitals. 3. *The gel-precipitin tests. Preparation of agar.* Agar was prepared by immersing commercially available agar (Difco) in several volumes of cold distilled water, and incubating at refrigerator temperature. After the agar had settled, the water was decanted and the sediment resuspended in fresh water. After 5 such changes, swelling of the agar began and the washing was terminated. The sediment was now collected by centrifugation and lyophilized. It was now white in color.

Technic of the tests. The following 2 procedures were employed: Single diffusion in 1 dimension: The procedure was described by Oudin, in which antigens diffuse from an overlying solution into a serum-agar gel(3). Double diffusion in 1 dimension: This method, described by Oakley(4), was used with modifications suggested by Preer(5). The technic involves 3 layers: antigen solution, an agar solution, and serum, from above downwards. In this procedure the antigens and antibodies each diffuse into the agar column. Bands of turbidity occur in the agar where any given antigen-antibody system is within the zone of equivalence, with a sufficiently high concentration of the antibody to cause visible precipitation. Soft glass tubes, acid cleaned, of 3 mm internal diameter and 10 cm length, were sealed at one end, lined with 0.3% washed agar solution, described by Oudin(3), and marked on the outside at 2, 3, and 5 cm from the bottom. Serum was added to the tube to the 2 cm mark (0.13-0.14 cc of serum). Bubbles were removed if they formed. Agar, in 0.3% solution containing merthiolate 1:10,000, which had been heated and then maintained at 48-52°C, was added slowly over the serum layer up to the 3 cm mark. The tubes were allowed to stand 15-20 minutes at room temperature to ensure complete solidification of the agar. The antigen solution, generally used at optimum concentration of 3 mg cc and containing 1:10,000 merthiolate, was added up to the 5 cm mark. The tubes were sealed with adhesive tape, placed in a glass jar or desiccator, and stored in a cabinet at room temperature. In the majority of sera tested, the maximum number of bands was present on the third day. The test was read in a darkened room with the tube held against a fluorescent back light. The number of bands was determined, and a brief physical description of each band noted. Three grades of relative density of bands could be readily differentiated: C—clear, distinct, dense band, with sharp boundaries; F—faint, distinct, band with sharp boundaries, but not dense; VF—very faint band with clear boundaries. These gradations of density reflect relative concentrations of the respec-

FIG. 1. Photograph of gel-precipitin tubes of 3 sera tested against concentrates of streptococcal culture supernate. Each pair of tubes is derived from a single serum, tested by single diffusion in the tube on the left and by double diffusion on the right. In all cases the meniscus constitutes the bottom of the antigen column. Note the greater resolution and the lower position (relative to the meniscus) of the bands in the case of the tubes set up by double diffusion (9,8,10).

tive antibodies at the level of the band, since the amount of specific precipitate reflects largely concentration of antibody. All bands not otherwise marked were of the dense type.

Fig. 1 shows gel-precipitin tubes of 3 sera tested by both single and double diffusion, on the third day of the test.

Results. 1. *Gel-precipitin tests with sera of rabbits injected with streptococcal CSA.* Rabbits given such courses of injections showed considerable variation in antibody response. Of those injected with alum-precipitated material only a few developed anti-CSA complement-fixation titers greater than 32, or more than 2 or 3 bands in the gel-precipitin tests, even after a number of courses of injection. Of the rabbits injected with the solution of CSA a higher percentage developed complement-fixation titers above 100 after the first 2 or 3 courses of injections, and at least 3 gel-precipitin bands within that time. Examination of the sera through the period of successive courses of injection with the CSA showed a general sequence of increasing complement-fixation titers and numbers of gel-precipitin bands against CSA from the pre-injection levels (CF titer below the threshold of measurement, and no gel-precipitin bands), although a number of instances of disparity occurred. Thus, one animal showed only 2 very faint bands and a complement-fixation titer of 128 after the first course of injections, whereas another of that group at the same stage showed 3 bands by gel-precipitin with a complement-fixation titer of only 32. Of the entire group of animals, the best antibody response obtained corresponded to 5 bands in gel-precipitin tests and complement-fixation titers of 192-256.

2. *Tests with sera of presumably normal human adults.* Eighteen such sera were collected from the group of normal men. These subjects were approved as blood donors by recent medical history and superficial physical examination. Of 10 such sera chosen at random for gel-precipitin tests against streptococcal CSA, 2 showed 2 bands of precipitate, 7 showed 1 band and 1 showed none. Eight other sera were selected from a larger number on the basis of antistreptolysin and antihyaluronidase titers which were relatively high for the normal population (AH, 16-192; ASL, 64-256). Of these sera 1 showed 3 bands in the gel-precipitin test, 3 showed 2 bands, and 4 showed 1 band. No association was apparent between the level of the titers of the 2 neutralizing antibodies and the number of bands in the gel-precipitin test. Samples of 7 lots of gamma globulin prepared from pools of normal human sera, made available through the courtesy of the American Red Cross, were similarly tested. Of these,

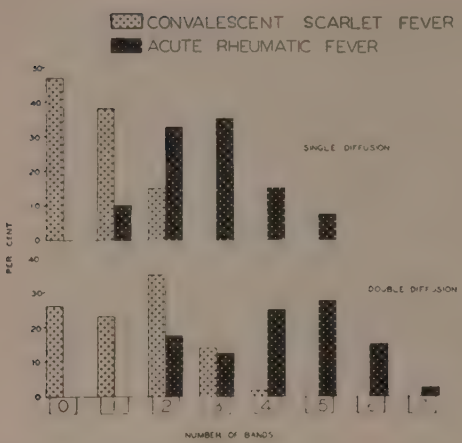

FIG. 2. Percentage distribution of sera of patients with acute rheumatic fever, and convalescent from scarlet fever, respectively, according to number of bands produced in tests with streptococcal culture supernates, by single-diffusion and double-diffusion technics.

1 sample yielded 2 bands, 5 gave 1 band, and 1 gave none. These tests were done by the single-diffusion method.

3. *Sera of patients convalescent from scarlet fever and patients with acute rheumatic fever.* A group of sera obtained from patients convalescent from scarlet fever were tested for antibodies to antigens present in the streptococcal CSA. A total of 65 such sera were tested by the double-diffusion technic, 34 of which were also tested by single diffusion. The number of bands which appeared by the third day ranged from 0-2 in the case of the tests by single-diffusion and 0-4 by double diffusion. Sera which were obtained from 40 patients with acute rheumatic fever were also tested. Using the single diffusion technic the number of bands which developed ranged between 1 and 5, and in tests by double diffusion the number of bands ranged between 2 and 7. The sera tested in each group were distributed according to the number of bands which appeared by each method, and percentages calculated. Fig. 2 presents the data obtained. A larger percentage of sera from patients with acute rheumatic fever developed relatively greater number of bands within this distribution in tests, by both technics. Of one of the sera of a patient convalescent from scarlet fever a larger specimen was available. Globulins were precipitated from a portion of this serum by adding an equal volume of a saturated solution of ammonium sulfate. The sediment was redissolved, and the solution dialyzed overnight and then brought to a volume equal to that of the original portion of serum. This solution yielded the same number of gel-precipitin bands as did the original serum.

4. *Sera of patients with scarlet fever at various times relative to the onset of the disease.* Of the sera of patients with scarlet fever a number were chosen whose convalescent sera had developed from 2-4 bands, for a comparison of the number of bands present early in the disease, at convalescence (3 weeks), and 6 weeks after the onset of symptoms. These were examined in a single test by double diffusion. The results are summarized in Table I. In all cases there was an increase in the number or intensity of the bands from the acute to the convalescent stage of this disease. In some cases, where bands were already present in the acute serum specimen, this difference was smaller. The relatively high antistreptolysin-O titers in some of these acute specimens would suggest, however, that some of these sera may have been obtained after the disease had been present long enough for some antibody response to have occurred. (It should be noted that the acute serum is obtained at the time of admission to the hospital, which is usually some days after the onset of symptoms. Also, the notation of day of disease on which the serum is obtained depended on the medical history provided by

TABLE I. Number of Gel-Precipitin Bands Produced by Sera from Patients with Scarlet Fever at Various Times in Relation to Onset of the Disease, Tested with Streptococcal CSA. (8 patients.)

Early serum specimen		No. of bands		
Day after onset of symptoms	Antistreptolysin titer	Acute	3-wk	6-wk
3	12	0	2	2 + 1F
3	256	1	2	2
4	64	0	4	1 + 2F
2	384	3F	3	2F
2	96	0	2	2
4	128	3F	3	2 + 1F
4	64	1WF	2	2
4	256	2	2 + 1F	3

TABLE II. Number of Gel-Precipitin Bands Produced by Sera of Patients with Acute Rheumatic Fever Observed over Periods Ranging from 4-18 Months. (18 patients.)

Acute serum	Months after onset 2	4	6	8	12	18
1 + 1F	3	2	2	1 + 1F	1 + 1F	1 + 1F
5F	1 + 1VF	1	1			1
4	2	1 + 1F	1 + 1VF	1F	1F	
5	1 + 1F	1F	1F	1VF	1VF	
3	2	2F			1 + 1F	
4	1 + 2F	2VF			1VF	
3	1 + 2F				1 + 1F	
7	1 + 1F	3F	2	1 + 1F		
5	1 + 1F	2	2	1 + 1VF		
3	2	1 + 1F	1F + 1VF	1F + 1VF		
5	2	3		2 + 1VF		
3	1 + 1F	1 + 1F	1			
3	2	2	2			
4 + 2VF	6	5	3 + 2VF			
6	3	2				
5	1 + 1VF	1				
6	3 + 3F	3				
5 + 1F	4F					

a parent.) In the sera obtained 6 weeks after the onset of symptoms no increase in the number of bands was found over the 3-week serum specimen, and occasionally a decrease in the number of bands was noted.

5. *Sera of patients with rheumatic fever at different stages of the disease.* A group of patients with rheumatic fever was studied over the period from acute disease to quiescence. For each patient, sera which were obtained in the acute stage and 2, 4, 6, 8, 12 and 18 months later were tested by double diffusion. There was a definite decrease in the number of bands, from the period of acute rheumatic fever to inactive rheumatic fever. In many instances some decrease in the number of bands was already evident 2 months after the acute attack. Table II summarizes the findings obtained in 18 patients with acute rheumatic fever followed for 4 to 18 months. Sera obtained in an out-patient clinic from 12 persons with inactive rheumatic fever were tested by the double-diffusion technic. Of these, 1 serum showed 3 bands; 3 sera 2 bands; 5 sera 1 band; and 1 serum no bands.

Discussion. Comparison of data obtained by single-diffusion (Oudin) and double-diffusion technics. In all groups of sera, both by single and double diffusion, the latter technic resulted in more bands, for the reason that in the double-diffusion method the concentration of any antibody varies over a continuous range which extends to zero, whereas in the single-diffusion method the concentration of an antibody is never below a given fraction of its concentration in the serum.† A technical advantage of the double-diffusion technic is that it permits the use of turbid sera, or sera which have become turbid through freezing and thawing, since the observation of bands of precipitate is not made in the zone which contains serum. *Number of gel-precipitation bands obtained in various groups of sera tested with streptococcal culture supernate concentrates.* The data presented above indicate that there are a considerable number of antigenic components in concentrates of streptococcal culture supernates prepared for these studies. As many as 7 bands have been found in the case of single sera and the total number of antigenic species may be greater than that for 3 reasons. (1). Within any single tube the number of bands is a minimum

† In the case of single diffusion the concentration of antibody at the interface soon becomes one-half its concentration in the serum-agar column (i.e., one quarter its concentration in the original serum); concentration of antibody thus varies within the agar column from one-quarter to one-half its concentration in serum. In the case of the double-diffusion technic the concentration of any antibody within the agar column varies from zero, at the antigen interface, to one-half its concentration in the original serum, at the serum interface.

of the number of antigen-antibody systems present because the difference between rates of diffusion of 2 antigenic species may be too small for the limit of visual resolution of the corresponding bands; (2). identification of the bands with respective antigens is not possible at present, and we do not know that antigens from the same group of 7 are involved in all the bands observed in various sera; (3). there may be antigens to which the antibodies are not present in the rabbit or human sera in sufficient concentration to produce visible precipitate. Evidence that these bands are produced by precipitation of streptococcal antigens with homologous antibodies is offered by the fact that they appeared *de novo* in sera of rabbits injected with the streptococcal CSA, and by the time relation of numbers of bands found in the sera of patients with scarlet fever and rheumatic fever to the times of onset of the diseases.

The relationship of any of these antigens to the known extracellular enzymes of the hemolytic streptococcus is not known. There have been two reports of gel-precipitin studies of concentrates of streptococcal culture filtrates which had been partially purified with respect to a biologically active agent. Jennings(6) found 8 separate precipitin systems in preparations of streptococcal erythrogenic toxin tested by the Oudin technic against horse antitoxin, and identified one of these as the toxin itself. Recently Halbert *et al.* examined preparations of streptococcal hemolysin-O by using human and rabbit sera in the same technic and found as many as 4 bands. Here, again, evidence was presented that one of these was hemolysin, the other bands being due to other antigens, which had not been separated from the biologically active one for which partial purification had been made(7). Using a crude concentrate of streptococcal culture filtrate in which no separation of macromolecules had been attempted, these authors found as many as 7 bands in the sera of patients with acute rheumatic fever, and no more than 4 in sera of patients with miscellaneous diseases other than rheumatic fever (8). The data presented here on the numbers of bands obtainable in gel-precipitin tests between sera in acute rheumatic fever and crude streptococcal culture supernate concentrates thus agrees with those of Halbert *et al.*

Difference between distributions of gel-precipitation band numbers in sera of patients with acute rheumatic fever and those convalescent from scarlet fever. The presence of a band in gel precipitation reflects the presence of an antibody in a concentration above the threshold required to produce a visible precipitate. Accordingly, the differences in band numbers found between these 2 groups of patients reflect higher concentrations of serum antibodies to streptococcal antigens in acute rheumatic fever than in convalescence from an uncomplicated streptococcal infection. It is not possible at present to compare antibody concentration to the individual antigens contained in these preparations, since there is no way of identifying the bands due to the respective antigen-antibody systems. This suggestion of higher concentrations of antibodies to streptococcal antigens in acute rheumatic fever, in comparison with streptococcal infection, agrees with observations by various workers in the case of a number of antibodies to streptococcal antigens (neutralizing antibodies to extracellular enzymes and complement-fixing antibodies to somatic nucleoproteins)(9,10).

No explanation can be suggested for the difference in range of distribution of serum antibody concentration to streptococcal antigens in these two diseases. Data are available on only one aspect of the contact between streptococcal antigens and the tissues of the rheumatic host which might result in such a difference—the greater interval of time than 3 weeks which might elapse between the onset of a foregoing streptococcal infection and admission to a hospital for rheumatic fever of many of these patients. That mere duration of contact between antigens and host tissues is not the causative factor is suggested by the fact that in the case of the individual patients with scarlet fever tested at both 3- and 6-week intervals after the onset of the disease there was no increase in the number or intensity of bands at the 6-week interval, in comparison with the 3-week, but rather a decrease in the intensity of some of these.

Summary. 1. Precipitin tests in semi-solid

agar (gel-precipitin) have been carried out between concentrates of streptococcal culture supernates and sera of various sources. Two technics of diffusion in 1 dimension (tube) were used: the single-diffusion technic of Oudin, and a double-diffusion technic. 2. Sera of rabbits previously injected with streptococcal culture supernates showed increasing numbers of bands as the course of injections progressed. Sera of such rabbits showed as many as 5 bands when tested with such concentrates, indicating that this was the minimum number of antigen-antibody systems present. 3. Sera of patients with acute rheumatic fever and of patients convalescent from an acute streptococcal infection (scarlet fever) were also tested by this technic against concentrates of streptococcal culture supernates. The group with rheumatic fever showed a greater number of bands (a range of 2-7) than did the group with scarlet fever (0-4), when tested by the double-diffusion technic. The corresponding ranges found with the single-diffusion technic were 1-5 and 0-2, respectively. In a sampling of sera from convalescents from scarlet fever the number of bands was found to be no greater 6 weeks after the onset of the infection than 3 weeks after the onset.

1. Harris, T. N., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 33.

2. Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., Jr., *J. Bacteriol.*, 1942, v43, 495.

3. Oudin, J., *Methods Med. Research*, 1952, v5, 335.

4. Oakley, C. L., and Fulthorpe, A. J., *J. Path. and Bact.*, 1953, v65, 49.

5. Preer, J. R., personal communication.

6. Jennings, R. K., *J. Immunol.*, 1953, v70, 181.

7. Halbert, S. P., Swick, L., and Sonn, C., *J. Exp. Med.*, 1955, v101, 539.

8. ——, *ibid.*, 1955, v101, 557.

9. Harris, T. N., Harris, S., and Nagle, R. L., *Pediatrics*, 1949, v3, 482.

10. McCarty, M., in *Rheumatic Fever, a symposium*, Thomas, L., ed., pp 135-149, University of Minnesota Press, Minneapolis, 1952.

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Teratogenic Effects of *Lathyrus odoratus* Seeds on Development and Regeneration of Vertebrate Limbs.* (21934)

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Diets containing *Lathyrus odoratus* seeds fed to young rats produce characteristic defects of mesodermal structures(1). Chemical analysis of extracts from the seeds points to the β-aminopropionitrile (BAPN) as the active fraction of the teratogenic principle(2-5). However other aminonitriles(5) and β-mercaptoethylamine(6) when given to rats have been reported to produce similar lesions. The Lathyrus poisons gain in interest through the similarity of the induced lesions with certain skeletal and vascular diseases in man, such as slipped epiphyses, degenerative arthritis, and dissecting aneurism of the aorta. Therefore the use of aminonitriles in animal research seemed to offer an opportunity for the elucidation of these human diseases.

We reported(7) that BAPN at a concentration of 2 μl/liter of aquarium water produces characteristic deformities in Xenopus larvae. The high sensitivity of Xenopus larvae suggests their use for the detection and quantitative evaluation of lathyrism-inducing agents. The effect of BAPN appears to be highly specific, affecting particularly certain rapidly developing mesodermal tissues. In view of the similarity between the processes of embryogenesis and of regeneration, the studies were extended also on the restitution of amputated legs in the newt, *Diemictylus viridescens*.

Methods. In a *first series* of experiments

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TABLE I. Effects Produced by Lathyrus Seeds, β-aminopropionitrile, and Other Compounds in Xenopus Larvae (Witschi's Stage 28). Class 1-4 express degrees of severity, 0 indicates normal development.

Compound	Conc., μl/liter	No. of animals	Class
Powdered seeds*		20	2
Sweet pea water extract†		20	2
Acetonitrile	20	10	0
Propionitrile	20	10	0
α-aminopropionitrile	10	10	0
β-aminopropionitrile	10	20	4
	2	20	3
	1	20	2
	.5	20	1
	.2	20	0
Aminoacetonitrile (hydrosulfate)	10	10	0
γ-aminobutyronitrile	10	10	0
Acrylonitrile	10	10	0
Glutamine	20	10	0
Potassium cyanide	10	10	0
Controls		100	0

* About 1 g of powdered seeds per day was placed in each aquarium; larvae were given no other food.

† Extract of 2.5 g of dry seeds per liter of water.

larvae of the South African toad, *Xenopus laevis*, were exposed to the natural principle by feeding powdered Lathyrus seeds *ad lib.* or weak solutions of BAPN,† or solutions of other related compounds. BAPN is a liquid and mixes with water freely; therefore concentrations are expressed in μl per liter of aquarium water. Since preliminary experiments had shown that treatments of early stages are without effect on limb development, the here reported experiments were all started with nearly fullgrown larvae, 10 to 12 days before the expected breakthrough of the forelimbs. At this stage the legs are still short and not yet movable. Lots of 10 to 20 larvae were placed in aquaria containing 5 liters of dechlorinated water. In the *second series* of experiments, hen's eggs (White Rock breed) were injected with 20, 1, .2 or .1 μl of BAPN in saline. Penicillin was added at the rate of 100 units per injection. The injections were made into the yolk sac either on the fourth or the tenth day of incubation, and the eggs were candled to observe the embryos daily thereafter. In the *third series* of experiments, a hind limb of adult newts was amputated at the middle of the thigh. Immediately after the wound had healed 10 animals were placed in water containing BAPN at a concentration of 2 μl/liter; and 10 served as controls.

† Supplied through the courtesy of Dr. S. Wawzonek, who synthesized the compound from acrylonitrile and ammonia.

Results. A. *Experiments with Xenopus larvae* (Table I). BAPN in concentration of 10 μl/liter causes death of the animals during metamorphosis. The limb deformities are most severe and frequently subcutaneous hemorrhages occur in this group. The larvae exposed to lower effective concentrations complete their metamorphosis, but they are too weak to survive beyond this stage. Fig. 1 represents a metamorphosing tadpole from a lot receiving BAPN at 2 μl/liter together with a control. The legs of tadpoles treated with this concentration are so badly twisted that it is impossible to prepare longitudinal sections for histological studies. At all levels of concentration the dislocation of the knee and ankle joints represents a most characteristic deformity (Fig. 2). A loosening of leg muscles, tendons and ligaments is clearly noticeable under a binocular microscope after removal of the skin. The microscopic examination of animals treated only 5 to 6 days shows wide clefts between cartilage and the covering connective tissue in the region of the joints (Fig. 3). In control animals of corresponding stage a compact embryonic connective tissue surrounds the cartilages and connects adjoining epiphyseal ends (Fig. 4). Obviously this rent formation is the beginning of the lesions in the young toads, a process which eventually results in dislocation of the joints. The cartilage appears normal and the periosteal ossification seems unaffected.

Lesions also develop in the dorsal musculature about 10 days following the onset of the experiments. After removal of the dorsal skin of tadpoles treated with BAPN or with Lathyrus seeds one observes that the intersegmental septa are abnormally wide (Fig. 1a, b). Detailed microscopic examination shows a very loose connective tissue in the septa. The muscle segments are atrophic.

The general body growth of the treated larvae becomes increasingly retarded. Experimental animals are thinner and weaker

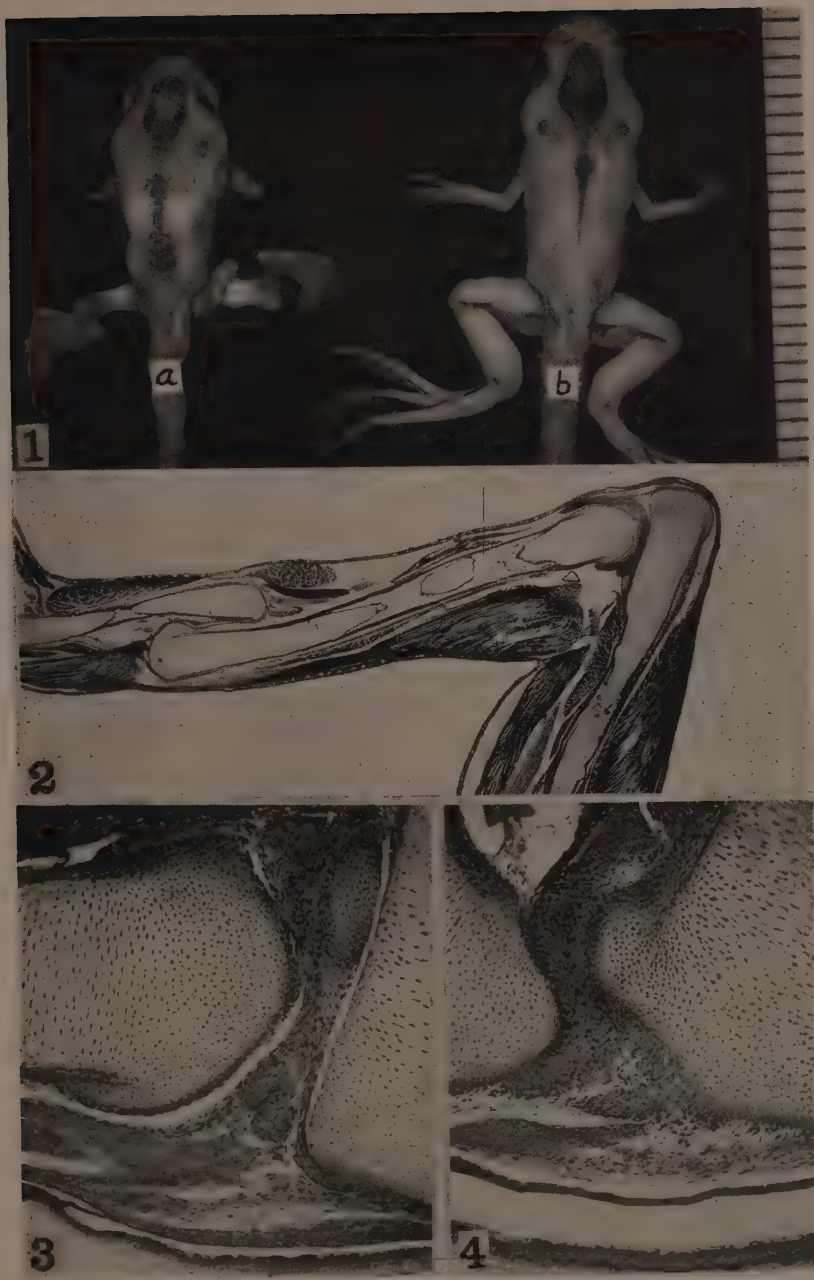


FIG. 1. a. Xenopus larva treated for 12 days with BAPN at a concentration of 2 μl/liter. Skin removed, showing dorsal musculature and widened intersegmental septa. b. Normal control of same age, skin removed.

FIG. 2. Longitudinal section through a hind leg of a Xenopus larva treated with BAPN for 10 days; concentration: 1 μl/liter; $\times$ 12.

FIG. 3. Section through the knee joint of a young tadpole, stage 29, fed with sweet pea seeds, showing the presence of clefts between the connective tissue and the epiphysis; $\times$ 120.

FIG. 4. Section through the knee joint of a control, stage 29; $\times$ 120.

TABLE II. Teratogenic Effects of BAPN on Chick Embryos.

Dosage, μl/egg	No. of eggs	No. died	Survived	Effect
20	13	13	0	Lethal
1	24	24	0	"
.2	20	13	7	Limb lesion
.1	10	6	4	" "
Control*	16	4	12	0

* Saline and penicillin.

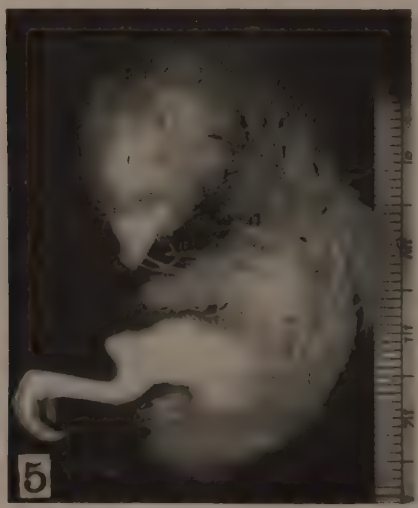
than the controls, probably because food intake is impaired. The minimal dose producing noticeable effects is less than ,5 μl/liter. It is noteworthy that within each dosage group all animals, without exception, were affected similarly.

It is of interest that treatments started after metamorphosis remain ineffective even if continued over periods of 2 months. Only slight deformity of the hind-legs developed in a few animals that had been treated over a period of 3 months with a high concentration of BAPN (4 μl/liter). However, no dislocation of joints ever occurred.

All compounds other than BAPN so far tested gave negative results. The high potency of aminoacetonitrile in producing skeletal lesions in rats as reported by Wawzonek *et al.*(5) could not be verified in tadpoles. Injections of α-tocopherol (total dose: 50 mg per tadpole) into the body cavity of tadpoles treated with BAPN did not protect the animals from developing the lesions. This result differs from that reported by Lee for the rats(8).

B. *Deformities produced in chick embryos.* In order to test the effect of BAPN on embryos of warmblooded animals, incubating hen's eggs were injected with BAPN. Since the embryonic development of the chick takes place within a closed egg shell, permitting no elimination, quantitative assays may be expected to give results of highest accuracy. The experiments are summarized in Table II. BAPN has a lethal effect at dosage levels of 1 μl and more per egg. The lesions produced in lower dosage groups are highly specific, affecting particularly the development of the legs (Fig. 5, 6). Size and weight of the animals are the same as those of the controls. Whether injected on the fourth or the tenth day, a bending of the femur end of the primary ossification center appears on the thirteenth day of incubation (Fig. 6). No dislocation of joints was observed. Cartilage and bones are histologically normal.

C. *Deformities in regenerating limbs.* Newts were used for this investigation because



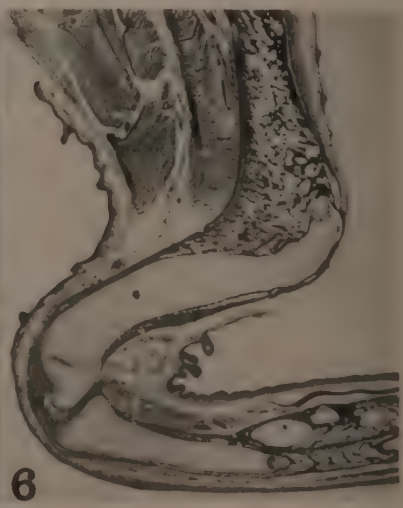


FIG. 5. Chick embryo injected with .2 μl BAPN; age 17 days of incubation.
FIG. 6. Longitudinal section through a leg of a 13-day chick embryo injected with .2 μl BAPN; × 15.

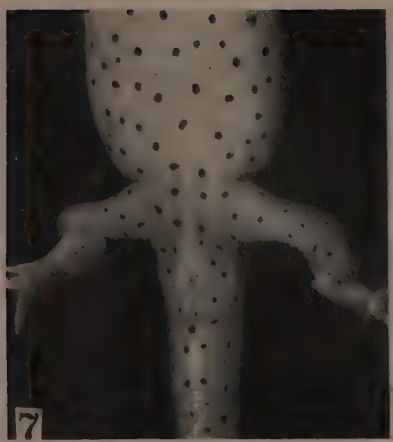


FIG. 7. Newt treated with BAPN during process of leg regeneration; concentration: 2 μl/liter. Left side intact limb, normal; right side, regenerated limb, showing dislocation of joint; $\times$ 2.

FIG. 8. Longitudinal section through regenerated limb in above figure. Note severe displacement at joint and atrophy of muscle; $\times$ 10.

FIG. 9. Longitudinal section through a normal regenerated limb; $\times$ 10.

of their well-known capacity of regeneration. After amputation of a hind limb it regenerates almost completely within about 3 months. The results of BAPN treatments are in principle as in the foregoing experimental series (Fig. 7). Microscopically the bones appear dislocated (Fig. 8, 9). Studies of serial sections also indicate atrophy of the leg muscles and loosening of the tendinous insertions.

Discussion. The results of the present series of studies indicate that BAPN is the teratogenic agent of Lathyrus seeds. In earlier experiments it was observed that 10 μl/liter of BAPN did not visibly affect cleavage and gastrulation of Xenopus eggs. However, the dorsal musculature and the skin of the hatched larvae were severely damaged. Further studies on the effect of myotome development are in progress. At the present it may be concluded that BAPN affects a definitely limited stage of the process of muscle and tendon differentiation. The action of this compound is tissue specific, affecting mainly the mesodermal tissues, probably the intercellular ground substance; but it is not species specific, producing similar lesions probably in all vertebrates. The severe teratogenic effect on embryonic and regenerating tissues suggests that the formation or normal activity of some essential morphogenic agent is inhibited by the presence of this compound.

Attempts at comparing the value of amphibians and of birds as bioassay animals are made difficult by the difference in the mode of administration of the compound. The chick as well as the amphibians are highly responsive and might be used for assay purposes in clinical work.

Summary. The evidence presented indicates that β-aminopropionitrile is the teratogenic agent of the seeds of *Lathyrus odoratus*. It produces dislocation of joints and loosening of intersegmental septa in amphibians, and bending of the femurs in chick embryos. The minimal teratogenic concentration for Xenopus larvae is .5 μl/liter, the minimal dose for chick embryos is .1 μl per egg. The effects are highly tissue specific but not species specific. The available evidence suggests that a single morphogenic process is interfered with by the presence of this compound during differentiation.

1. Ponseti, I. V., and Shepard, R. S., *J. Bone and Joint Surgery*, 1954, v36A, 1031.

2. Dupuy, H. P., and Lee, J. G., *J. Pharm. Assn., Sci. Ed.*, 1954, v43, 61.

3. Schilling, E. D., and Strong, F. M., *J. Am. Chem.*

Soc., 1954, v76, 2848.

4. Dasler, W., *Science*, 1954, v120, 307.

5. Wawzonek, S., Ponseti, I. V., Shepard, R. S., and Wiedenmann, L. G., *ibid.*, 1955, v121, 63.

6. Dasler, W., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 196.

7. Chang, C. Y., Witschi, E., and Ponseti, I. V., *Anat. Rec.*, 1954, v120, 816.

8. Lee, J. G., *J. Nutrition*, 1950, v40, 587.

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Histochemical Studies of Effects of Cortisone on Fetal and Newborn Rats.*† (21935)

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There is a growing body of experimental evidence demonstrating the effect of cortisone on enzyme systems(1-3). These investigations, for the most part biochemical in nature, have been carried out on a variety of cellular enzymes(4-6). Much emphasis has been placed on the effect of cortisone on alkaline phosphatase. A number of workers(7), utilizing histochemical methods, have observed an increase in the alkaline phosphatase content of the brush border of the kidney tubule after injection with cortisone. Moog(8-10), obtained a precocious increase of intestinal phosphatase upon injection of fetal or newborn mice with cortisone. She interprets these results as indicating that cortisone influences the basic metabolic processes which are responsible for phosphatase synthesis.

Ribonucleic acid (RNA) has also received much attention as a substance involved in cortisone action. Decreases in hepatic RNA after cortisone injection have been observed by several investigators(11), although there is some conflict on this point(12,13). One group(7) noted an increase in renal tubule RNA after cortisone injection.

In view of the widespread interest in the mode of action of cortisone, it was decided to extend and correlate the above-cited findings. Because of the importance of the functional relationships existing between alkaline phosphatase, RNA and polysaccharide-protein complexes, histochemical methods were employed to follow the changes induced by cortisone on these materials.

Materials and Methods. Two series of animals were employed. The first series consisted of 24 female Wistar rats. Each female was placed with a male and daily vaginal smears were made until fertilization occurred (as evidenced by sperm in the smear). Beginning with the seventh day of pregnancy, the mothers were divided into 3 equal groups. Group I animals received 75 mg of cortisone per kilo of body weight daily throughout pregnancy; Group II animals received 100 mg and Group III served as controls. The cortisone was divided into 2 daily doses, given 8 hours apart.

Each of the above groups was subdivided into 2 sub-groups of equal size. In one Caesarian sections were performed on the fourteenth day and in the other on the sixteenth day of gestation. The uterine horns were exposed under moderate nembutal anesthesia, a tally of the number of fetuses present was taken and one-half of the number was removed. The incisions were then closed and

* This investigation has been supported, in part, by a grant-in-aid from the Dental Research Institute of the U. S. Public Health Service to Dr. E. D. Goldsmith.

† Cortisone acetate (Cortone, Merck) used for these experiments was generously supplied by Merck and Co., Rahway, N. J.

‡ Based in part upon a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University.

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cortisone treatment was continued. A second Caesarian section was performed 4 days after the first, in each case yielding 18 and 20 day fetuses respectively. All fetuses were weighed on a torsion balance and fixed *in toto* at 4°C for 24 hours in Bouin's, Carnoy's or 95% ethyl alcohol. A total of 201 fetuses was so obtained.

In the second series of experiments, a total of 110 post-partum rats was used. Litters were kept with the mother and beginning with 24 hours after the hour of birth, the litter mates were divided into 2 groups. The first group received a daily injection of 0.1 mg of cortisone while the second group served as vehicle-injected controls. Injections continued until 24 hours before autopsy. At least 2 complete litters were autopsied on each of the following days of age: 3, 5, 7, 9, 11, 13, and 15. Liver, intestine and kidney were fixed as in the first series.

In addition to a routine hematoxylin and eosin stain, the following histochemical methods were employed. Alkaline phosphatase was demonstrated by the Ca-Co method of Gomori(14). Both DNA and RNA were visualized by methyl green-pyronin Y used according to the methods of Taft(15) and Kurnick(16). To substantiate the specificity of the stain, alternate sections were digested with a solution of ribonuclease (Worthington) in distilled water. Mucopolysaccharides were identified by the periodic acid-Schiff method of McManus(17). Glycogen was identified by malt diastase digestion.

Results. As was to be expected, at all ages the cortisone-injected animals showed a significant inhibition of weight gain as compared to the controls. This was evidenced by a marked smallness in size of the fetal rats and by a general debilitated appearance in the post-partum rats. There was no difference in response between the fetal rats whose mothers received the 75 mg dosage and those of mothers receiving the 100 mg dosage.

Fetal Rats. In the fetal rats, beginning with the 16th day of intra-uterine life, the hepatic parenchyma of the cortisone injected animals showed increases in glycogen deposition and decreases in RNA content when compared with controls of the same age. This has been a constant finding in all previous studies in this area(11-13). No differences became observable in the intestinal epithelium and renal tubule epithelium of the hormone-treated animals until the 16th day of intra-uterine life. At this age, there is the beginning of a response to hormone treatment which becomes accentuated at 18 days of age. By the 18th day, there is a higher RNA content in the supra-nuclear regions of the intestinal epithelium in the hormone-treated fetuses than in the controls of the same age. This increase of RNA is even more pronounced at 20 days of age, where the controls show only a moderate RNA content (Fig. 1a) while the experimentals demonstrate quite marked accumulations of RNA (Fig. 1b).

Similarly, in the brush border of the renal tubules there is an increased deposition of RNA in the cortisone-treated animals. This increase begins at 16 days of age and is quite pronounced at 18 days; the controls showing only a moderate RNA brush border content (Fig. 2a) while the treated animals demonstrate heavy deposits in the equivalent loci (Fig. 2b). This difference is no longer evident at 20 days of age.

As far as PAS-positive materials are concerned, the only significant differences are found in the renal tubule epithelium of the 18-day-old fetuses. In this site, there is evidence of increased glycogen deposition in the treated fetus (Fig. 3b) as compared with the control (Fig. 3a).

Along with these increases in RNA, concomitant increases in alkaline phosphatase were evident. Signs of increased alkaline phosphatase activity of the striated border could be seen in the 18-day-old treated fetuses. This heightened enzyme activity in the cortisone injected animal was more outstanding at 20 days of age. In the control animal of this age, the intestinal striated border shows only a slight alkaline phosphatase activity (Fig. 4a). The hormone treated animal, on the other hand, exhibits a marked activity which extends into the supra-nuclear cytoplasm (Fig. 4b). Both of these slides were incubated in the substrate for 10 minutes.

In the renal tubule epithelium, the cortisone treated animals exhibit an increased alkaline

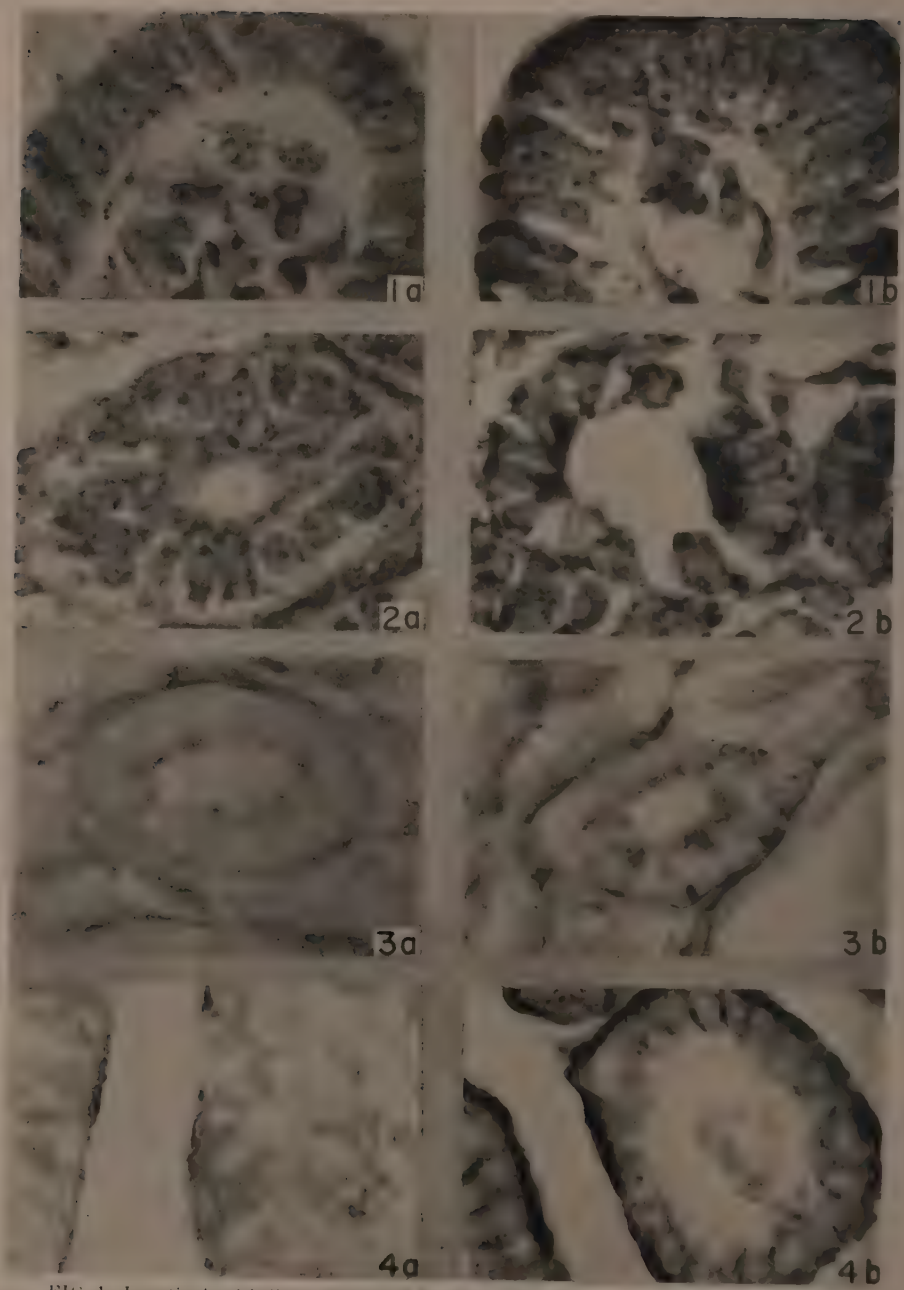

FIG. 1. Intestinal epithelium, 20-day old fetal rat. Methyl green-pyronin. a, control; b, experimental. Note marked deposition of supra-nuclear RNA in treated fetus. ×600.

FIG. 2. Renal tubule, 18-day-old fetal rat. Methyl green-pyronin. a, control; b, experimental. Note increase in brush border RNA in b. ×600.

FIG. 3. Renal tubule, 18-day-old fetal rat. PAS. a, control; b, experimental. Note deposition of glycogen in and adjacent to brush border in b. ×600.

FIG. 4. Intestinal epithelium, 20-day-old fetal rat. Alkaline phosphatase, ten min. incubation time. a, control; b, experimental. Note in treated fetus (b) greater alkaline phosphatase activity as compared to control (a). ×600.

phosphatase content beginning with 16 days of age. This difference is even more exaggerated at 18 days. At this time, the controls show very little brush border alkaline phosphatase activity (Fig. 5a). The treated animals, on the other hand, demonstrate a very marked enzyme activity in the same site (Fig. 5b).

Post-partum Rats. In the post-partum rats, the cortisone injected animals at all ages exhibited a marked increase in hepatic glycogen deposition and a decrease of RNA content. As far as RNA is concerned, while the controls showed heavy deposits of RNA, usually in the form of rodlets or spheroids (Fig. 6a), the experimental animals demonstrated only scattered remnants of this material (Fig. 6b).

As far as the intestine and kidney are concerned, no effects of cortisone treatment were observed until the 9th day of age. At this time, significant increases in alkaline phosphatase appeared in the treated animals. This became more marked in the 1-day-olds (the oldest age group that survived the cortisone treatment). There were no such corresponding increases in RNA and PAS-positive materials. The intestinal epithelium of the 11-day-old control animals showed a moderate striated border alkaline phosphatase activity (Fig. 7a). Treated animals of the same age, on the other hand, show a more marked activity in the same site (Fig. 7b). The renal tubule brush border activity is similarly enhanced, the controls showing only moderate alkaline phosphatase deposition (Fig. 8a), while the experimental animals demonstrate a strong activity (Fig. 8b).

Discussion. Essentially, the results herein presented are in accord with the findings of Karnofsky and his group(18) on the injection of cortisone into the chick embryo and with Moog's(6-8) experiments on the influence of cortisone on the fetal and post-partum mouse.

In our work, we have observed that there were no histochemically demonstrable effects of exogenous cortisone administration in the fetus until the 16th day of intra-uterine life. Karnofsky and his group noted that the chick embryo did not show a significant response to cortisone administration until after the 10th day of development. They maintained that the cortisone, in its observable effects, influences the utilization of a new substrate which is provided for cell growth as a result of the shift in embryonic metabolism occurring at this stage. The same line of reasoning might be applied to the present work on the rat, wherein a sensitive indicator of cortisone action, the liver, showed no response until the 16th day of fetal life.

Our observations have indicated that the RNA, alkaline phosphatase and glycogen of the renal tubule brush border are increased in the treated fetus as compared with the controls beginning with the 16th day and that this difference is no longer in evidence by the 20th day. The striated border of the intestinal epithelium of the treated animals did not show any increases in alkaline phosphatase and RNA until the 18th day. This increased response, however, was obtained through the 20th day. In both the renal tubule and intestinal epithelium the cortisone served to bring about a precocious acquisition of RNA.

In the post-partum rats a similar picture was observed. A response of the intestinal and renal tubule epithelium to cortisone could not be demonstrated in rats younger than 9 days of age. As in the fetuses, the response was exhibited as a precocious increase in the amounts of alkaline phosphatase and RNA. Moog has suggested that the intestinal epithelium passes through a transitory state of sensitivity to cortisone as a stage in its normal developmental sequence. The addition of excess exogenous cortisone merely hastens the basic metabolic response processes involved in the functional differentiation of both the intestinal and renal tubule epithelium.

Thus, there would appear to be 2 stages of sensitivity to cortisone in the rat. The first

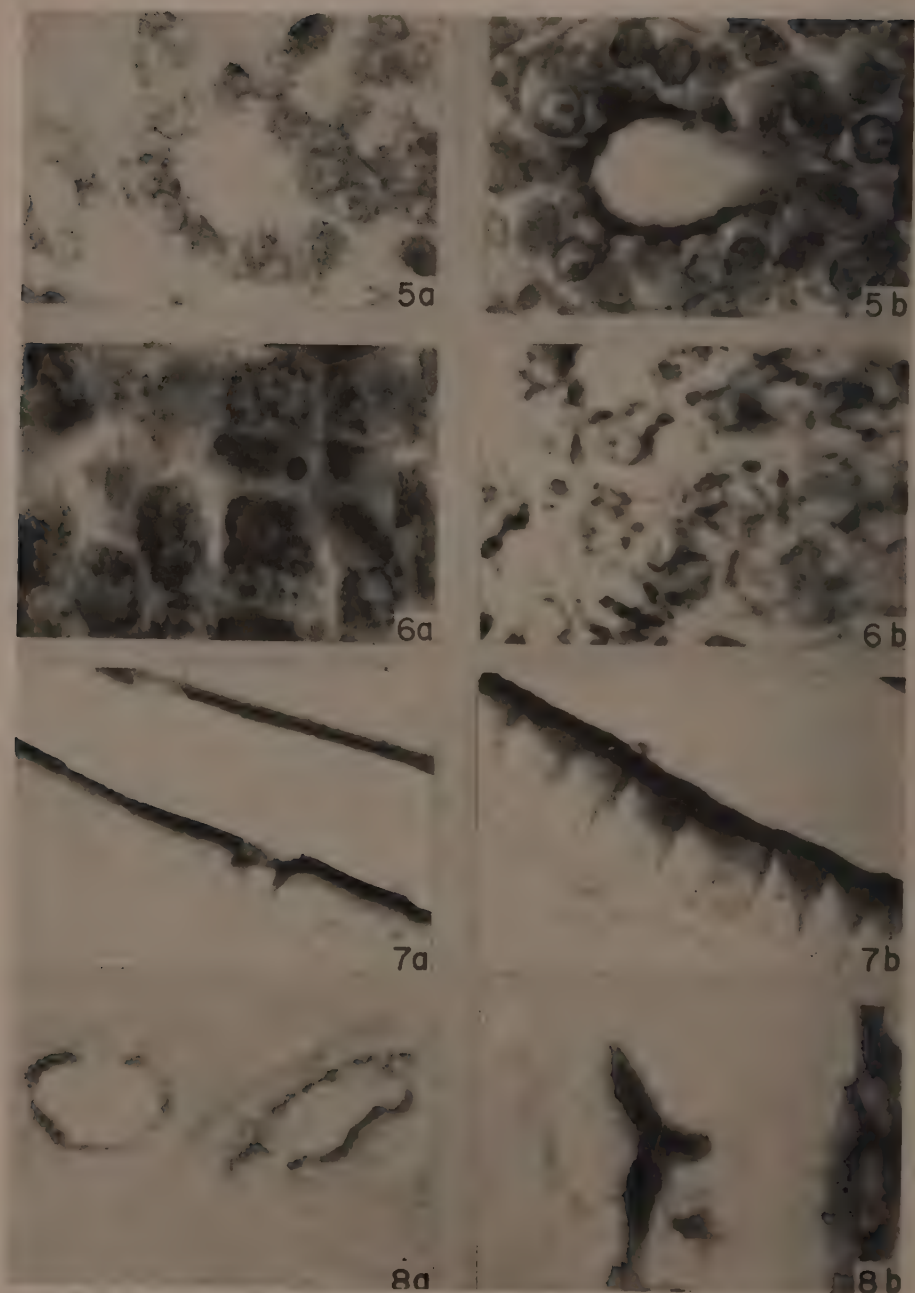

FIG. 5. Renal tubule, 18-day-old fetal rat. Alkaline phosphatase, 10 min. incubation time. a, control; b, experimental. Note very strong enzyme reaction in treated fetus (b) and contrast this with control (a). ×600.

FIG. 6. Liver, 11-day-old post-partum rat. Methyl green-pyronin. a, control; b, experimental. Marked depletion of RNA is very prominent in b. ×600.

FIG. 7. Intestinal epithelium, 11-day-old post-partum rat. Alkaline phosphatase. Ten min. incubation time. a, control; b, experimental. Treated rat (b) shows a strong enzyme reaction evident even in supra-nuclear cytoplasm. ×600.

FIG. 8. Renal tubule, 11-day-old post-partum rat. Alkaline phosphatase, 10 min. incubation time. a, control; b, experimental. Note greater brush border phosphatase activity in b. ×600.

appears in the embryo at 16 days of fetal life for the kidney tubule and at 18 days for the intestinal epithelium. The former is lost by the 18th day, whereas, the latter is still evident at 20 days. In the post-partum rat, the stage of sensitivity first appears at 9 days of age for both kidney and intestinal epithelia.

Baker and Bridgman(19), who studied the gastric and intestinal mucosa in adrenalectomized adult rats, noted a decrease in cytoplasmic RNA and striated border alkaline phosphatase activity. Following the injection of cortisone into these animals the RNA was reconstituted but the amount of alkaline phosphatase present was not affected. These findings lend further support to our observations as well as those of Moog(9) that after a certain stage of development cortisone is apparently no longer able to influence the formation of alkaline phosphatase in the intact animal.

It should, however, be emphasized that had Baker and Bridgman used whole adrenocortical extract, they might have altered the alkaline phosphatase activity in the adrenalectomized animal.

Summary. A histochemical study of the effect of cortisone on the liver, intestine and kidney of fetal and post-partum rats was made. There were no histochemically observable effects of cortisone on the fetus until the 16th day of age. Beginning with this day it was found that cortisone caused an increase in glycogen deposition and a decrease in RNA content of the hepatic parenchyma. In the post-partum rats these liver effects were noted at all ages. The intestinal striated border demonstrated a precocious acquisition of alkaline phosphatase and RNA as compared to controls of the same age. In the renal tubules these effects were manifest in the 16- and 18-day-old fetuses, while the intestinal effects were obtained in the 18- and 20-day-old fetuses. Similar precocious increases in renal and intestinal alkaline phosphatase and RNA were noted in the cortisone-treated newborns beginning with the ninth day of age.

1. Spicher, R. R., *McGill Med. J.*, 1951, v20, 191.
2. Umbreit, W. W., *Ann. N. Y. Acad. Sci.*, 1951, v54, 569.
3. Verzar, F., *Vitamins and Hormones*, 1952, v10, 297.
4. Kochakian, C. D., and Vail, V. N., *J. Biol. Chem.*, 1944, v156, 779.
5. Dorfman, R. I., *Vitamins and Hormones*, 1952, v10, 331.
6. Kerpolla, W., *Endocrin.*, 1952, v51, 192.
7. Herlant, M., and Timiras, P. S., *Acta Anat.*, 1952, v14, 65.
8. Moog, F., *J. Exp. Zool.*, 1951, v118, 187.
9. ———, *ibid.*, 1953, v124, 329.
10. Moog, F., and Thomas, E. R., *Endocrin.*, 1955, v56, 187.
11. Williams, W. L., *et al.*, *Anat. Rec.*, 1953, v115, 247.
12. Fraenkel-Conrat, J., and Li, C. H., *Endocrin.*, 1949, v44, 481.
13. Gros, F., Bonfils, S., and Macheboeuf, M., *Compt. Rend. Acad. Sci.*, 1951, v233, 990.
14. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 23.
15. Taft, E. B., *J. Exp. Cell Res.*, 1951, v2, 312.
16. Kurnick, N. B., *Stain Tech.*, 1952, v27, 233.
17. McManus, J. F. A., *ibid.*, 1948, v23, 99.
18. Karnofsky, D. A., Ridgway, L. P., and Patterson, P. A., *Endocrin.*, 1951, v48, 596.
19. Baker, B. L., and Bridgman, R. M., *Am. J. Anat.*, 1954, v94, 363.

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Electrolyte Balances Following Total Body X-Irradiation. (21936)

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One of the prominent features of irradiation sickness is the attendant upset of salt and water metabolism. Depending upon the species, this may be manifested in the forms of vomiting, diarrhea, and diuresis. An exact determination of the changes in electrolyte balance following total body x-irradiation is, therefore, of interest.

Methods. Twenty 250 g male white rats (Rolfsmeyer Co., Madison, Wisconsin) were placed in metabolism cages over siliconized glass funnels, and allowed free access to distilled water and a diet containing 60% sucrose, 18% lactalbumin, 16.9% Crisco, 4% modified Wesson salt mixture (minus its Na^+, K^+, and Cl^-), 1% liver extract (Wilson Co., liver fraction D, Lot 41863), 0.1% choline hydrochloride, with supplements of thiamine, pyridoxine, riboflavin and calcium pantothenate. Roughly 5% of the Na^+, K^+, and Cl^- intake was furnished by the diet; coming largely from the liver extract. The remainder of the sodium and potassium chlorides was administered daily by intraperitoneal injection. In the control period the average total electrolyte intake was 1.55 meq of sodium, 2.37 meq of potassium, and 2.54 meq of chloride per 48-hour period. Under these conditions the electrolyte intake was largely independent of the level of food intake. Each 48 hours, the food intake and body weight were recorded, and the excreta were removed for analysis. The excreta were collected quantitatively in barium hydroxide solution, evaporated to dryness, and ashed at 500°C for 12 hours. Sodium and potassium determinations were made on food, injection solutions, and excreta, with a flame photometer attachment to the Beckman DU Spectrophotometer. Chlorides were determined on the same samples by the Volhard method. After 18 days of control study, 16 of these animals were given a total body dose of 700 r of x-ray (140 kv., 0.25 mm Cu, 1 mm Al). In preliminary tests, with larger groups of animals, this dose was found sufficient to kill half of the animals within a 2-week period. Balance studies were continued for an additional 10 days following irradiation. Four of the animals maintained in metabolism cages were used as parallel controls. They received no x-irradiation, but otherwise were handled in the same manner as the experimental animals. Balance data obtained from these controls were used as a base-line in the interpretation of the data obtained from the irradiated animals. The figures in Table I represent the difference in balance between the experimental and the control groups at each period. In this way it was possible to minimize any effect due to handling at the time of irradiation, and to compensate for the fact that the 300 g male white rat was still gaining weight. This procedure also tends to minimize the effect of any consistent bias in analytical methodology.

TABLE I. Difference between Electrolyte Balances of Experimental and Control Rats before and after Total Body X-irradiation.

Pre-X-ray days	K^+	Na^+	Cl^-
0- 2	+.050	+.180	+.124
2- 4	−.093	+.145	+.050
4- 6	−.081	+.127	+.014
6- 8	+.156	+.031	+.047
8-10	−.113	+.061	+.168
10-12	+.162	+.107	+.214
12-14	+.016	+.196	−.077
14-16	−.100	−.121	−.007
16-18	−.136	−.008	−.364
Avg =	−.015	+.080	+.019
σ	.146	.101	.169
Post-X-ray days			
0- 2	−.567†	−.245*	+.066
2- 4	−.866†	−.558†	+.124
4- 6	−.112	+.045	−.424*
6- 8	−.033	−.178*	−.143
8-10	+.214	+.312	+.183

* Significant at 5% level ($.01 < P \leq .05$).
† " " 1% " ($P < .01$).

Results. In Table I it is seen that the potassium, sodium, and chloride balances are significantly altered following total body x-irradiation with an LD_{50} dose of x-ray. There

is a marked loss of potassium during the first 4 days following irradiation, with a gradual recovery to a positive value by 8-10 days following irradiation. The changes in sodium balance are roughly parallel with those in the potassium balance data. Quite a different picture is seen in the chloride data, however. The chloride balance remains close to zero for the first 4 days of the post-irradiation period, and only after the 4th day is there a significantly negative balance. Again, there is evidence of recovery in the later periods.

The data of Table I represent the means of over 800 independent determinations carried out in triplicate. The control day-to-day standard deviations, representing the over-all analytic and physiologic variations, are only 5-7% of the intake. This degree of uncertainty is even further decreased by the large amount of control data available for comparison. The changes observed under these conditions are statistically significant ($P<.05$ for chloride and $P<.01$ for sodium and potassium), and clearly define the nature of the post-irradiation electrolyte balance changes in the rat exposed to an LD_{50} dose of total body x-irradiation.

Discussion. From previous reports in the literature, it can be inferred that following irradiation the body electrolyte balances become positive(1-3), negative(4-7), or are uninfluenced(8). Unfortunately, none of these reports presents balance results which are statistically significant.

The main features of the post-irradiation electrolyte balances, as defined in Table I, are: (A) a prompt (0-4 day) loss of K^+ and Na^+, (B) a delayed (4-6 day) loss of Cl^-, and (C) a recovery of electrolyte beginning at the 8-10 day period.

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1. Bennett, L. R., Bennett, V. C., and Howland, J. W., *Fed. Proc.*, 1949, v8, 350.
2. Cameron, A. T., and McMillan, J. C., *Lancet*, 1924, v2, 365.
3. Cori, C. F., and Pucher, G. W., *Am. J. Roentg.*, 1923, v10, 738.
4. Bowers, J. Z., and Scott, K. G., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 645.
5. Edelmann, A., *Fed. Proc.*, 1949, v8, 39.
6. Glenn, J. L., and Bass, W. R., *ibid.*, 1954, v13, 55.
7. Soberman, R. J., Keating, R. P., and Maxwell, R. D., *Am. J. Physiol.*, 1951, v164, 450.
8. Bowers, J. Z., Davenport, V., Christensen, N., and Goodner, C. J., *Radiology*, 1953, v61, 97.

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Studies on the O Antigen of *Salmonella typhosa* IV. Endotoxic Properties of the Purified Antigen. (21937)

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Toxic manifestations induced in man and experimental animals by Gram-negative bacteria have been established as referable to their production of a distinctive component, variously described as somatic antigen, endotoxin, pyrogen, and tumor hemorrhagic agent. These specific attributes of endotoxins have been described, particularly by Burrows(1), Bennett and Beeson(2), and Thomas(3). Endotoxins are generally referred to as complexes of polysaccharide, lipid, and protein; however, the precise chemical composition of the complex in relation to endotoxic activity has not been established. Biological properties of endotoxins have been studied for the most part with relatively crude preparations, frequently culture filtrates or extracts(4). Those purified products in recent investigations(5-9), contained significant quantities of nitrogen, which probably represented appre-

ciable protein or polypeptide components. Thus, the essentiality of the protein moiety for biological activity remains obscure.

Our papers described isolation of the somatic antigen of *Salmonella typhosa* as a lipopolysaccharide containing only 0.6% nitrogen, and showed that despite the essentially complete removal of protein, its activity as an antigen was not diminished, but increased (10,11). This report shows that this product possesses the properties characteristic of the classical endotoxins. Inasmuch as the biological effects of endotoxins are well-documented, this report is restricted to a summary of data on endotoxic activities of the lipopolysaccharide derived from *S. typhosa*.

Materials and methods. Endotoxin: The product was isolated by Dr. M. E. Webster, from viable *S. typhosa*, 0-901, by an ethanol-ammonium sulfate fractionation procedure. The purified endotoxin is a phosphorylated lipopolysaccharide containing only 0.6% nitrogen. Its chemical and immunological properties have been described(10,11). *Pyrogenicity tests*: Female New Zealand white rabbits weighing approximately 3 kg were employed. Animals free of demonstrable typhoid O agglutinins were utilized. They were quartered in individual cages in animal room maintained at 75°F. Rectal temperatures were obtained with clinical mercury thermometers. Normal or baseline temperatures were obtained several days prior to use. Following administration of test material, temperatures were taken hourly for 5-7 hours. Rabbits were not used for more than a single test.

Results. General toxic properties: The purified lipopolysaccharide derived from *Salmonella typhosa* possessed, to a high degree, the toxic attributes of colonially smooth Gram-negative bacilli. When administered intravenously to susceptible host, such as the rabbit, exceedingly small amounts were capable of inducing fever, leucopenia, diarrhea, prostration, and death. The activity of the purified endotoxin in eliciting this toxic syndrome is as follows:

Lethal Activity: The lethal action of intravenously administered endotoxin for rabbits is shown in Table I. Despite control of

TABLE I. Lethality of Typhosa Endotoxin for Rabbits.*

μg endotoxin (i.v.)	No. of animals dead / Total animals inj.
10	2/10
20	7/10
50	6/10
100	3/10
500	8/15

* New Zealand white females weighing 2.1-2.4 kg.

strain, sex, weight, and age of rabbits, considerable variation in susceptibility of individual animals to the lethal action of the endotoxin exists. It is significant that administration of endotoxin over a wide range of dosage in no instance resulted in 100% mortality. On several occasions, the percentage of rabbits succumbing to large doses of endotoxin (100 to 500 μg) was less than that obtained with 20-50 μg. At present there is no explanation for this paradox. Despite this apparent lack of dose-response relationship, a large series of individual tests in this and in related studies have shown that the lipopolysaccharide generally, but not invariably, is lethal for rabbits in dose range of 20-50 μg.

As observed by other workers(1), we found that in contrast to the rabbit, the mouse is relatively refractory to endotoxin. While much larger doses (on the basis of body weight) are required, the lethal action of intraperitoneally injected endotoxin in this species was more reproducible and consistent than in the rabbit. Thus, the LD_{50} for 15 g Bagg albino mice was approximately 250 μg. Like the mouse, the guinea pig tolerated relatively large doses of endotoxin. The LD_{50} for this species was somewhat more variable and lay in the range of 500-1000 μg.

The lethal activity of the lipopolysacchar-

TABLE II. Lethality of Typhosa Endotoxin for Mice. Effect of alkali treatment.

μg endotoxin (i.p.)	Endotoxin	Alkali-degraded endotoxin
200	7/10*	0/10
400	8/10	0/10
600	6/10	0/10
1000	10/10	0/10
2000		3/10

* Fractions equal No. of animals dead of total animals injected.

TABLE III. Pyrogenic Activity of Typhosa Endotoxin. Comparison with intact typhoid bacilli.

Preparation	Dose (μg)	Pyrogenic response of individual rabbits (°F)				Mean temp. rise (°F)	M.P.D.* (μg)
S. typhosa 0-901	.3	2.6	1.4	1.7		1.9	.015
	.03	2.0	1.1	.8		1.3	
	.003	.4	.7	.1		.4	
Purified typhosa endotoxin	.008	2.2	2.9	3.0	3.1	2.8	.0002
	.0016	1.3	2.1	1.6	1.9	1.7	
	.00032	1.5	1.3	1.2	1.1	1.2	
	.00006	.4	.6	.5	.3	.4	

* Minimum pyrogenic dose: Quantity giving rise to 1°F elevation in temperature.

ide was considerably decreased following autoclaving, boiling for 30 minutes, or exposure to weak alkaline hydrolysis (*i.e.* 0.02 N NaOH for 18 hours at room temperature). A comparison of the lethal action for mice of alkali-degraded endotoxin with undegraded endotoxin is illustrated in Table II. On a weight basis, the product subjected to alkaline hydrolysis exhibited less than 10% of the lethal activity of the untreated product.

Pyrogenicity. During investigations on the chemical and immunological properties of the typhosa endotoxin, it was observed that intravenous injection of rabbits with 0.01 μg or less of this lipopolysaccharide resulted in rapid production of fever, associated with leucopenia and subsequent leucocytosis. Generally, temperatures rose within an hour after administration of the product, reached a maximum at approximately 3 hours, and gradually declined to normal in 6 to 8 hours. A marked neutrophilic leucopenia was observed one hour after injection, and the total leucocyte count gradually rose to normal at 4-6 hours, followed by a leucocytosis. These alterations in temperature and leucocyte counts were primarily dependent on the dose of lipopolysaccharide administered. The remarkable pyrogenic activity of this lipopolysaccharide is shown in Table III where the response of groups of rabbits to graded doses of this product are compared with those of animals receiving acetone-killed and dried typhoid bacilli. When the mean temperature response is plotted against dose of lipopolysaccharide administered, the response is rather well related to dose over the range shown in this Table. It is apparent that the purified product is a far more potent pyrogen than the whole organism. The quantity of this component present in intact typhoid bacilli of the 0-901 strain has been reported as 3.3% by Staub and Combes(12), and 6.2% by Webster *et al.*(10). These data indicate that the isolated component should be approximately 15-30 times as active as the bacterial cell. The finding that it is in fact somewhat more active suggests that greater availability may favorably influence pyrogenic activity.

A state of insusceptibility to the pyrogenic action of this endotoxin could be developed in rabibts by a series of injections of the lipopolysaccharide. A group of 10 rabbits treated with 10 daily injections ranging from 1-20 μg (initial dosage 1 μg was progressively increased at 2-day intervals), exhibited little or no thermal response to 1 μg of endotoxin 24 hours after the last injection (mean temperature rise of 0.6°F). This is in contrast to the mean temperature rise of 4°F. developed following injection of 1 μg on the first day of the experiment. On the other hand, a constant daily dose of 1 μg administered to a second group of 6 rabbits for a period of 15 days, did not result in as significant a depression of the fever peak obtained after the initial injection. After 1, 2, 3, 10, and 15 injections, mean temperature rises were 4.6°, 3.5°, 2.9°, 2.6° and 2.4° respectively. In addition, this peak was attained relatively earlier as the injection series progressed.

Animal species were found to vary greatly in their pyrogenic response to this product. Thus, the administration of 10 μg of lipopolysaccharide intravenously brought about a marked thermal response in 2 horses and 2 dogs. On the other hand, during an 8-hour

period, no rise in temperature was observed to occur in groups of mice, rats, guinea pigs, or chicks injected intravenously with 10 μg of this product. Three 20-25 lb chimpanzees were given a single intravenous injection of 1 μg of lipopolysaccharide. A mean elevation of 2.7°F ensued and the leucocyte count fell to approximately one-half the normal values shortly after injection, followed by a 3-4 fold increase in the number of these cells during a period of 24 hours. The pyrogenic response of man to intravenous injection of the purified endotoxin has not yet been determined.† However, in a recent study of its antigenicity in man(13), it was observed that a subcutaneous injection of 20 μg frequently, but not invariably, resulted in erythema, induration, fever, and malaise, while edema and lymphadenitis were encountered occasionally. On the whole, the reactions exhibited by the purified endotoxin were somewhat more marked than those induced by the conventional typhoid vaccine.* These more pronounced reactions might be expected since the quantity of purified endotoxin administered was greater than that calculated to be contained in the standard dose of 0.5 ml of typhoid vaccine (7.5 μg).

Activity in Eliciting the Shwartzman Reaction. Local Reaction: A quantitative study was made of the activity of purified endotoxin as a preparing and provoking agent for the phenomenon of local skin reactivity in the rabbit. Groups of animals were prepared for this reaction with amounts of endotoxin varying from 100 to 0.5 μg. Dilutions of 0.2 ml of this product were injected intradermally at 4 sites on the clean-shaven abdominal skin of each animal. Rabbits, so prepared, were divided into groups of 5-12 animals, and 20 hours later each group was provoked with an intravenous injection of lipopolysaccharide ranging from 100 to 0.2 μg. The ability of individual animals of the same sex, size and strain to respond to this component with development of the characteristic local reaction varied; however, in those rabbits capable of responding the incidence of reactivity followed a decreasing gradient as either the preparatory or provocative dose was reduced (Table IV). As would be expected, the extent and severity of the lesions tended to be minimal when the area had been prepared or provoked by the smallest quantities of endotoxin. Nonetheless, it is apparent that as little as 1 μg, or even less, was sufficient to prepare and provoke this reaction.

TABLE IV. Quantitative Assessment of Activity of Typhosa Endotoxin in Production of Local Shwartzman Reaction.

Provocative dose (μg) admin. i.v. 20 hr after preparatory dose	Preparatory dose (μg) inj. intraderm.					
	100	25	10	5	1	.5
100		80*	60	40	40	
25	83	100	57	57	44	10
20	67	50	50		29	
15	67	33	0		0	
10	40	14	14	0	0	
5	40	40	40		20	
1	60				10	10
.2	10				0	0

* Figures indicate % of rabbits exhibiting positive reaction sites as indicated by visible hemorrhage and purpura. Groups of 5-12 New Zealand white rabbits (3 kg) were used.

The generalized Shwartzman reaction was induced with purified endotoxin from *S. typhosa* in young (2-3 lb) New Zealand white rabbits. Gross and histopathologic examination† of the characteristic lesion, bilateral renal cortical necrosis, showed that the pathology evoked by this purified component was in all respects similar to the classical changes induced by crude bacterial products (*e.g.* occlusion of small blood vessels of kidney with an amorphous fibrinoid-like material. The resultant punctate hemorrhagic and necrotic areas of the cortex occasionally extended into the medullary area) as described by Thomas and Good(7,8). The results of tests in which it was attempted to determine quantitatively

* Adsorption of the lipopolysaccharide on aluminum hydroxide resulted in complete suppression of these toxic manifestations. However, groups of 60 subjects each, inoculated with 20 μg of adsorbed endotoxin showed relatively poor antibody response (mean O agglutinin titer 1:26) in contrast to those of individuals receiving endotoxin as a saline solution (mean O agglutinin titer 1:90).

† The authors are indebted to Lt. Col. Helmuth Sprinz, Walter Reed Army Hospital, who prepared and examined sections of kidneys obtained from rabbits in this study.

TABLE V. Activity of Typhosa Endotoxin in Production of Generalized Shwartzman Reaction.

Preparatory dose of endotoxin (μg)	Hr elapsed between doses	Provocative dose of endotoxin (μg)	No. of animals exhibiting bilateral renal cortical necrosis of total inj.*
20	18	50	0/6
20	12	50	4/6
20	8	50	1/2
20	6	50	1/2
10	12	50	1/3
5	12	50	0/4
20	12	100	2/2
20	12	20	3/5
10	12	20	3/4†
10	12	10	2/4†
5	12	10	0/4

* Reaction evident in the gross 18 hr after provocation.

† Degree of reaction was considerably less in these animals.

the amount of endotoxin necessary to prepare and provoke this pathology in the rabbit are given in Table V. The principal variables in these experiments were preparatory dose, provocative dose, and time interval between the 2 injections. The data obtained indicated that production of this reaction does not follow a clear-cut dose-response relationship, but appears to be associated primarily with quantities of endotoxin not far removed from dosage shown to be lethal for the rabbit. The optimal time interval between the two injections, required for production of the characteristic kidney lesion, appeared to be somewhat less than that previously reported by Thomas and Good(7), who employed meningococcal washings as endotoxin.

Discussion. General toxic manifestations associated with administration of typhoid bacilli have been reproduced by a protein-free lipopolysaccharide isolated from an O variant strain of *S. typhosa*. For the most part, these studies have been conducted in the rabbit, a species highly susceptible to endotoxin. However, various animal species differ in their response to the endotoxic action of this product. Thus, man, horse, dog, and rabbit reacted strongly to the pyrogenic and antigenic stimulus of this product. On the other hand, it may be significant that species such as the mouse, rat, guinea pig, and chick, which did not exhibit a thermal response to injection of 10 μg of lipopolysaccharide, were the species previously shown to be refractory to this product as an antigen(11). Examination of sera from the latter group by the highly sensitive bacterial agglutination test showed no evidence of production of O antibody following this injection. However, it is not known (and it is difficult to obtain acceptable experimental evidence) whether a causal relationship exists between these two properties. In this connection, it is noteworthy that alterations in the lipopolysaccharide which resulted in decreased antigenic activity were accompanied by reduction in pyrogenicity(11). This lipopolysaccharide possessed antigenicity of a high order in the rabbit, *e.g.* a single intravenous injection of 0.001 μg elicited production of significant levels of agglutinins(11). The present study shows that in terms of one of the characteristic properties of endotoxin, namely pyrogenic action, this product exhibits extraordinary potency. It is difficult directly to compare and evaluate the pyrogenic potency of this product with that of the various fever-producing agents reported in the literature; nonetheless, the finding that the minimum pyrogenic dose for a 3 kg rabbit is approximately 0.0002 μg affords evidence that this typhosa lipopolysaccharide is one of the most potent pyrogens known. It has been shown that these attributes of pyrogenicity and antigenicity in the rabbit are manifested in essentially the same range of concentrations and exhibit similar extinction values(11). Depending on the species involved, lipopolysaccharide evokes either both effects, or neither. These findings may therefore be indicative of a common functional mechanism. Experimental evidence tending to support the thesis that these physiological activities of endotoxin may be responsible for marked antigenic activity, subsequently expressed by the host, was obtained in our studies on enhancement of antibody response to proteins by typhosa lipopolysaccharide(14).

The remarkable activity of this purified bacterial component in eliciting these experimental host reactions may provide further insight into the classical reactions encountered clinically in those diseases of man caused by

endotoxin-bearing organisms. For example, calculations based on rhamnose content and on actual isolation, indicated that 1 billion organisms (*S. typhosa*, 0-901) can contribute approximately 15 μg of lipopolysaccharide. That the minimum pyrogenic dose is approximately 0.0002 μg/3 kg rabbit, makes it apparent that endotoxic material released from relatively few bacteria (1×10^4) can produce fever in the rabbit. Man is considered to be more susceptible to endotoxin than the rabbit (15), and this study offers further evidence for the hypothesis advanced by Favorite and Morgan that under conditions of natural infection, extremely small quantities of endotoxin may elicit the pyrexial response in the typhoid fever patient.

A similar situation may apply in the Shwartzman reaction. It is apparent that rabbits vary in ability to react to the endotoxin used by us. The dose-response relationship of an individual rabbit is such that if susceptible to this reaction, it will respond over a wide range of concentrations of endotoxin. That nonsusceptible rabbits exhibit no reduction in their refractory state even when doses of endotoxin are greatly increased further attests to the "all or none" nature of this phenomenon. A comparable individual hyperreactive state has been postulated by Black-Schaffer *et al.* as being a prerequisite for the occurrence in man of the pathology characteristic of the Shwartzman reaction(16).

Summary. A study was made of the endotoxic properties of a protein-free lipopolysaccharide isolated from the 0-901 strain of *S. typhosa*. The product was lethal for rabbits in a dose range of 20-50 μg while 300-500 μg were required to kill mice. The minimum pyrogenic dose in rabbits was 0.0002 μg, characterizing it as a pyrogen of extremely high potency. In a quantitative study of the local Shwartzman reaction, 1 μg or less, was effective as a preparatory or provocative dose. Its activity in eliciting bilateral renal cortical necrosis, the lesion characteristic of the generalized Shwartzman reaction, was in the range of 5-20 μg. Thus, very small quantities of a lipopolysaccharide, freed of protein, are capable of producing the various biological alterations generally attributed to endotoxins.

Addendum. Since this report was submitted for publication preliminary tests of the effects of the typhosa lipopolysaccharide in man were undertaken(17). These indicate that when injected i.v. the minimum pyrogenic dose for a 70 kg subject lies in the range of .01-.04 μg.

1. Burrows, W., *Ann. Rev. Microbiol.*, 1951, v5, 181.

2. Bennett, I. L., and Beeson, P. B., *Medicine*, 1950, v29, 365.

3. Thomas, L., *Ann. Rev. Phys.*, 1954, v16, 467.

4. Shwartzman, G., *Phenomenon of Local Tissue Reactivity*, Paul B. Hoeber, Inc., New York, N. Y., 1937.

5. Morgan, H. R., *Am. J. Path.*, 1943, v19, 135.

6. Delaunay, A., Boquet, P., Lebrun, J., Lehoult, Y., and Delaunay, M., *J. Phys.* (Paris), 1948, v40, 89.

7. Thomas, L., and Good, R. A., *J. Exp. Med.*, 1952, v96, 605.

8. Good, R. A., and Thomas, L., *ibid.*, 1952, v96, 625.

9. Creech, H. J., and Hankwitz, Jr., R. F., *Cancer Research*, 1954, v14, 824.

10. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G., *J. Immunol.*, 1955, v74, 455.

11. Landy, M., Johnson, A. G., Webster, M. E., and Sagin, J. F., *ibid.*, 1955, v74, 466.

12. Staub, A. M., and Combes, R., *Ann. Inst. Pasteur*, 1951, v80, 21; 1952, v83, 528.

13. Landy, M., Gaines, S., Seal, J. R., and Whiteside, J. E., *Am. J. Pub. Health*, 1954, v44, 1572.

14. Johnson, A. G., Gaines, S., and Landy, M., *Fed. Proc.*, 1954, v13, 499.

15. Favorite, G. O., and Morgan, H. R., *J. Clin Invest.*, 1942, v21, 589.

16. Black-Schaffer, B., Hiebert, T. G., and Kerby, G. P., *Arch. Path.*, 1947, v43, 28.

17. Sanford, J. P., to be published.

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Some Effects of ω-Methyl Analogues of Vitamin B_6 in Rats. (21938)

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ω-Methylpyridoxine (2-ethyl-3-hydroxy-4, 5-bis(hydroxymethyl)pyridine), in which the methyl group of pyridoxine has been replaced by an ethyl group(1), was one of the first analogues of vit. B_6 to be synthesized and tested in living organisms. Robbins found the compound replaced pyridoxine in promoting growth of excised tomato roots(2), but was toxic for *Ceratostomella ulmi*(3). Corresponding analogues of pyridoxal and pyridoxamine also have been synthesized(4). For lactic acid bacteria such as *Streptococcus faecalis,* these latter compounds replace pyridoxal as growth factors under some conditions(4-6), but may inhibit growth under slightly different conditions, depending upon the vit. B_6-dependent process which limits growth(5,6). Like ω-methylpyridoxine(7), they inhibit growth of yeast (*Saccharomyces carlsbergensis*); this inhibitory effect is overcome completely by pyridoxine, pyridoxamine, or pyridoxal.

A single dose of ω-methylpyridoxine was less than 2% as effective as pyridoxine in alleviating symptoms of vit. B_6 deficiency in rats(1). No other reports of the physiological effects of these compounds in animals have appeared; results of such an investigation are presented below.

Materials and methods. Synthesis of ω-methylpyridoxal and ω-methylpyridoxamine has been described earlier(4). ω-Methylpyridoxine(1) and an authentic sample of the lactone of 4-pyridoxic acid(8) were gifts from Dr. Karl Folkers. Weanling albino rats were caged individually, and fed vit. B_6-deficient ration of Sarma *et al.*(9) modified by substituting vitamin-free casein (Labco) for blood fibrin. Supplements were added to the diet or given separately by stomach tube. In the latter event a tuberculin syringe and a dulled no. 15 needle, bent and cut off at the proper length, were used. Food and water were given *ad libitum.*

Results. a. Activity of ω-methylanalogues of vit. B_6 during preventive assay. Weanling rats (Sprague-Dawley) were fed the deficient diet supplemented wtih pyridoxal, one of the analogues, or both pyridoxal and analogue, as indicated in Table I. Over a 4-week period, the ω-methyl analogues possess considerable growth-promoting activity. However, the higher levels of analogues were no more effective than the lower levels, and no amount of analogue permitted a maximum growth rate. The compounds were not irreversibly toxic, for when fed together with pyridoxal, a high rate of growth was achieved.

When supplementation was continued for a longer time, it became evident that the high level of all 3 ω-methyl analogues permitted even less growth than the lower level. Such results are shown for ω-methylpyridoxal in Fig. 1. Eventually the rate of growth of supplemented animals slowed to that of the deficient controls. Thus, while the analogues permit growth for a limited length of time in animals containing initially adequate supplies of vit. B_6, they become unable to support growth once bodily supplies of vit. B_6 reach a sufficiently low level.

More striking evidence than growth rates, of the inability of these analogues fully to replace vit. B_6 was provided by the observed symptomatology. Beginning about 2 weeks after supplementation with the analogues, practically all animals of Groups 5 through 10, Table I, underwent frequent epileptiform fits, especially during weighing periods. Occurrence of such convulsions has been noted frequently in animals fed vit. B_6-deficient diets alone (*e.g.*10), but is not a prominent feature of vit. B_6 deficiency on this basal ration, and was not noted in any of the deficient animals of Group 1 (Table I) until fully 4 weeks *after* their occurrence had become common in the analogue-fed animals. Even then, the incidence of the attacks in deficient Group 1 was not more than 25% of that in analogue-fed animals. The 3 analogues, therefore, pro-

TABLE I. Comparative Effects of Pyridoxal and ω-Methyl Analogues of Vitamin B_6 on Growth of Rats. Each group contained 6 animals.

Group	Supplement	Amt of supplement/day, γ	Wt gain in 4 wk, g	Apparent molar activity (pyridoxal = 100)
1	0	—	46	
2	Pyridoxal	1.6	71	
3	"	4.1	94	
4	"	8.2	114	
5	ω-Methylpyridoxal	16.6	100	32
6	"	83	100	6.5
7	ω-Methylpyridoxamine	17.8	92	23
8	"	88	101	6.4
9	ω-Methylpyridoxine	16.6	100	32
10	"	83	93	5.1
11	Pyridoxal +	82	140	
	ω-methylpyridoxal	83		
12	Pyridoxal +	82	134	
	ω-methylpyridoxamine	88		
13	Pyridoxal +	82	126	
	ω-methylpyridoxine	83		

duce a deficiency of a type in which these fits are an unusually common feature—an observation of interest in view of the observed occurrence of convulsions in infants receiving suboptimal amounts of vit. B_6(11). No such attacks occurred in animals of groups 11-13, which received pyridoxal together with the high level of analogue.

b. *Activity of ω-methyl analogues of vit. B_6 during curative assay.* The apparent ability of the analogues to support growth only during the first few weeks (Fig. 1) suggests that as depletion of vit. B_6 proceeds, growth responses to the analogue would become less marked. This was checked by holding weanling animals (derived from the Wistar strain) on the deficient ration for 5 weeks, then supplementing the diet with high levels of ω-methylpyridoxine. A pronounced stimulation of growth occurred in the analogue-fed group during the first week, which decreased greatly in the course of the second and third weeks to a value below that of the deficient control group (Table II). Four of the 5 animals of the analogue-supplemented group died between the 34th and 41st days of supplementation, and the experiment discontinued. None of the deficient control animals had died during this period. This high level of ω-methylpyridoxine was non-toxic when fed together with pyridoxine. Thus the unusual result is observed that a compound that apparently corrects early deficiency symptoms eventually makes these symptoms more pronounced, and changes the intensity of individual symptoms relative to one another.

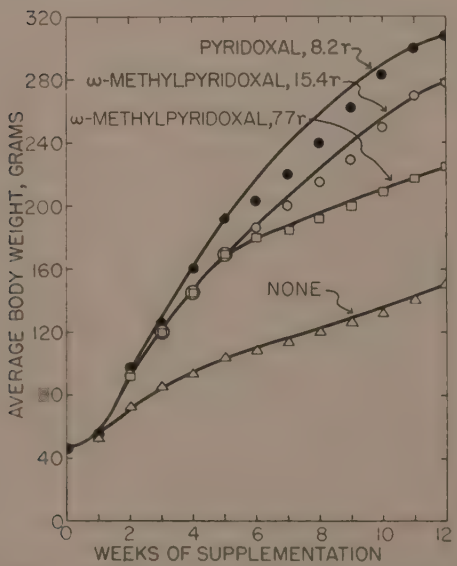

FIG. 1. Effect of time on comparative growth-promoting activities of pyridoxal and ω-methylpyridoxal for rats. Supplements indicated were administered daily.

c. *Metabolism of ω-Methylpyridoxine in Rats.* Application of the method of Heyl(8) for synthesis of 4-pyridoxic acid to a small sample of ω-methylpyridoxine yielded a product with an ultra-violet absorption spectrum essentially identical with that of 4-pyridoxic

TABLE II. Effect of ω-Methylpyridoxine on Growth and Survival of Vit. B_6 Deficient Rats. Each group contained 5 animals.

Supplement	Amt fed per day, γ	Avg wt gain/wk during 1st	2nd	3rd	4th	5th wk	Survivors at 41 days
0		3.2	1.3	.9	3.3	.5	5
Pyridoxine	8.2	27	16	15	16	8.3	5
ω-Methylpyridoxine	820	28	7.9	1.0	.1	−10.5	1

acid. After treatment in such a way as to form the lactone(12) the absorption spectrum proved identical with that of 4-pyridoxic acid lactone. On paper chromatograms the product migrated somewhat faster than 4-pyridoxic acid in all solvents, as might be expected from its slightly less polar character. The method of synthesis, the spectrum of the free acid, and the spectrum of the lactone, all demonstrate that the product is ω-methyl-4-pyridoxic acid. Different animals excrete variable proportions of administered pyridoxine as 4-pyridoxic acid(13); pyridoxal and pyridoxamine are also oxidized to this same product(14). To determine whether ω-methylpyridoxine was similarly metabolized, rats were fed high levels of ω-methylpyridoxine (4600 mμ moles day) of pyridoxine (4900 mμ moles/day) for 24 hours. Chromatographic comparisons (Table III) showed that administration of ω-methylpyridoxine gave rise to ω-methylpyridoxic acid in the urine. Quantitative determination by the method of Møller(12) showed, in agreement with results of Huff and Perlzweig(13), that only about 0.5% of administered pyridoxine was recovered as 4-pyridoxic acid; similarly, 0.6% of the administered ω-methylpyridoxine was recovered in the urine as ω-methyl-4-pyridoxic acid. In neither case are the other products of metabolism known.

TABLE III. Excretion of ω-Methyl-4-Pyridoxic Acid by Rats Fed ω-Methylpyridoxine. Approximately 30 mμmoles (.01 ml) of acids or lactones and 0.05 ml of urine samples were applied to 18¼ by 22½ inch sheets of Whatman No. 1 filter paper, and developed with butanol-ethanol-water (4:1:1) in an ammoniacal atmosphere. Zones of these fluorescent substances were detected by observation under ultra-violet light.

Substance chromatographed	R_f values Untreated	Treated for lactone formation
4-Pyridoxic acid	.68	.40
ω-Methyl-4-pyridoxic acid	.77	.53
Urine of pyridoxine-fed rats	.66	.44
Urine of ω-methylpyridoxine-fed rats	.77	.46

d. *Some Effects of ω-Methylpyridoxine Administration on Enzyme Levels in Tissues.* D-Amino acid oxidase, the glutamic-aspartic transaminase, and thionase (cysteine desulfhydrase) were selected as representative of one vit. B_6-independent and 2 vit. B_6-dependent enzymes and their levels in appropriate tissues from normal (Group 13, Table I), deficient (Group 1, Table I), and analogue-fed (Group 10, Table I) animals compared. Animals were killed by decapitation, the tissues excised and homogenized. Results (Table IV) show no diminution in activity of the vit. B_6-independent kidney D-amino acid oxidase, but demonstrate a moderate reduction in activity of liver transaminase and a severe reduction in liver thionase in both vit. B_6-deficient and analogue-fed animals. The differences in symptomatology observable in simple vit. B_6-deficiency and in analogue-fed animals thus cannot be explained in terms of the effects on these 2 enzymes, but must result from differential effects upon one or more of the multitude of other vit. B_6-dependent enzymes of tissues. *Rates* of diminution in enzyme activity, an effect not considered in the above comparisons, probably are also of importance here.

The result shows that the ω-methyl analogues are not the functional equivalent of vit. B_6 in activation of these enzymes, and if they are converted to ω-methylpyridoxal phosphate by animal tissues (as they are by bacteria (5,6)) the latter must be *comparatively* ineffective as an activator of these two enzymes. This was checked by comparing the effectiveness of ω-methylpyridoxal phosphate(4) and pyridoxal phosphate as activators of enzyme preparations obtained from rats fed the vit. B_6-deficient ration for 37 days. The analogue coenzyme activated liver glutamic-aspartic

TABLE IV. Comparison of Kidney D-Amino Acid Oxidase, Heart Glutamic-Aspartic Transaminase and Hepatic Thionase between Normal, Vitamin B_6-Deficient, and ω-Methylpyridoxine-Fed Rats. Each determination was carried out on pooled tissues of 3 animals. Normal and analogue-fed rats received vit. B_6-free diet and supplements for 86 days from weaning. Vit. B_6-deficient rats received the vit. B_6-free diet only for 49 days.

Treatment of animal	D-Amino acid oxidase,* μl O_2/hr/mg wet wt	Enzyme systems: Transaminase,† μl CO_2/10 min./mg wet wt	Enzyme systems: Thionase,‡ μg H_2S/2 hr/g wet wt
Normal	1.4	6.2	166
Analogue-fed	1.8	4.5	45
Vit. B_6-deficient	1.3	4.4	25

* By method of Armstrong, *et al.*(15).
† " " " Ames and Elvehjem(16).
‡ " " " Thompson and Guerrant(17).

transaminase, but was only 11% as active on the weight basis as pyridoxal phosphate. Liver thionase was not fully reactivated by any concentration of the analogue coenzyme; the partial reactivation achieved required over 50 times the concentration that sufficed when pyridoxal phosphate was the coenzyme. The behavior of these enzymes of mammalian origin to the analogue coenzyme resembles closely that of the corresponding bacterial enzymes(6).

Discussion. Under appropriate conditions in certain bacteria, ω-methylpyridoxal and ω-methylpyridoxamine support continued growth because they are converted to ω-methylpyridoxal phosphate, which activates the essential vit. B_6-dependent enzyme in place of pyridoxal phosphate(5,6). Under closely related conditions, when another set of vit. B_6-dependent enzymes is made limiting, the same vitamin analogues inhibit growth, presumably because these vit. B_6-dependent enzymes are not activated by the analogue coenzyme, which consequently behaves as a structural antagonist. That similar relationships hold in animal systems seems probable from the observations that ω-methylpyridoxine and pyridoxine are metabolized similarly, and that ω-methylpyridoxal phosphate varies in its ability to activate various vit. B_6-dependent enzymes of the rat. The observed facts that these analogues first promote, then inhibit, growth on vit. B_6-free rations might be readily interpreted in terms of these effects. It may be supposed that as the stores of vit. B_6 within the body become depleted, various vit. B_6-dependent enzymes become hypofunctional in turn, depending, upon their affinities for pyridoxal phosphate (*cf.* 18). If the first of these to become hypofunctional were also activated by ω-methylpyridoxal phosphate, this analogue would be expected to promote growth. When, however, additional essential vit. B_6-dependent enzymes that are not activated by the analogue coenzyme become hypofunctional, then the analogues no longer promote growth. Since the analogues act also as displacing agents, pyridoxal phosphate displaced by the analogue coenzyme from one apoenzyme may become available for activation of a second apoenzyme having different relative affinities for the coenzyme and analogue coenzyme.* This displaced pyridoxal phosphate may initially contribute to the growth effects of the analogue. At the same time, however, if the enzyme from which this pyridoxal phosphate has been displaced itself catalyzes reactions essential for growth, and is not activated (or is activated to an insufficient extent) by available concentrations of the analogue coenzyme, such displacement would explain the active toxicity exhibited by the analogues after more prolonged periods of administration. Since the enzymes from which pyridoxal phosphate is displaced are not necessarily the same as those that would lose this coenzyme first in simple vitamin deficiency,† administration of the analogue might be expected to produce a pattern of deficiency symptoms differing in some respects from those produced by simple deficiency, as is actually observed. Finally, a part of the vit. B_6 ingested by the animal normally is made unavailable for growth processes through oxidation to 4-pyridoxic acid (and unidentified products) and excretion. The participation of ω-methylpyridoxine as a competitive substrate in these latter reactions would decrease the amount of vit. B_6 destroyed in this way, and thus spare additional amounts for essential metabolic uses.

The similarity of these observations to

* That different enzymes of a single organism may show such varying affinities has been demonstrated in *Streptococcus faecalis*(5,6).

those made with "diethylriboflavin"(20) and with lyxoflavin(21) suggests a fairly widespread occurrence of similar phenomena.

Summary. ω-Methylpyridoxal, ω-methylpyridoxamine and ω-methylpyridoxine support growth of rats for several weeks following deletion of vit. B_6 from the ration. Eventually, however, growth rate in the presence of these compounds slows, and becomes equal to or less than that of vit. B_6-deficient control animals. The period of growth promotion is decreased greatly when the compounds are administered to depleted rats. Following this period of growth-promotion, growth with high concentrations of ω-methylpyridoxine is distinctly less than that in control animals; and death results well before any deaths are noted in the control group. In uncomplicated vit. B_6 deficiency on this ration convulsive seizures are infrequent and were observed only following approximately 6 weeks on the deficient diet. In contrast, in the animals fed the ω-methyl analogues of vit. B_6 convulsions occurred frequently, in all animals, starting about 2 weeks after supplementation while substantial weight gains were still occurring. All of these symptoms were prevented by feeding pyridoxal together with the analogues. Following administration of ω-methylpyridoxine to animals, ω-methyl-4-pyridoxic acid appears in the urine in amounts similar to those found for 4-pyridoxic acid following pyridoxine administration. Glutamic-aspartic transaminase and thionase are reduced in ω-methylpyridoxine-fed animals to about the levels found during uncomplicated vit. B_6 deficiency. Correspondingly, ω-methylpyridoxal phosphate was only about 11% as active as pyridoxal phosphate in activating the glutamic-aspartic transaminase, and did not fully activate liver thionase at any concentration. The facts that feeding these analogues first promotes, but eventually inhibits growth, and produces a deficiency of a different type than that found in uncomplicated vit. B_6-deficiency, may be interpreted in terms of (a) the dual character of ω-methylpyridoxal phosphate as an activator for some vit. B_6-dependent enzymes, but not for others, (b) its role as a structural antagonist for vit. B_6 in those enzymes that it does not activate, and (c) the role of these analogues as competitive substrates for vit. B_6 in reactions leading to destruction of the vitamin.

† Dietrich and Shapiro(19) have observed that the enzymatic disabilities produced by feeding 4-desoxypyridoxine to rats do not coincide exactly with those produced by simple vitamin deficiency. This result should be expected, since the degree to which an analogue displaces pyridoxal phosphate depends not only upon the dissociation constant of the various proteins for pyridoxal phosphate, but also upon their affinity for the analogue coenzyme and upon the rate at which the enzyme is inactivated and reformed within the tissues.

1. Harris, S. A., and Wilson, A. N., *J. Am. Chem. Soc.*, 1941, v63, 2526.

2. Robbins, W. J., *Am. J. Bot.*, 1942, v29, 241.

3. Robbins, W. J., and Ma, R., *Bull. Torrey Bot. Club*, 1942, v69, 342.

4. Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, 1954, v76, 637.

5. Olivard, J., and Snell, E. E., *J. Biol. Chem.*, 1955, v213, 203.

6. ———, *ibid.*, 1955, v213, 203.

7. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. and Biophys.*, 1952, v43, 408.

8. Heyl, D., *J. Am. Chem. Soc.*, 1948, v70, 3434.

9. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v165, 55.

10. Chick, H., El Sadr, M. M., and Worden, A. V., *Biochem. J.*, 1940, v34, 595.

11. Maloney, C. J., and Parmelee, A. H., *J. Am. Med. Assn.*, 1954, v154, 405.

12. Møller, P., *Acta Chem. Scand.*, 1951, v5, 1418

13. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1944, v155, 345.

14. Rabinowitz, J. C., and Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 235.

15. Armstrong, K. F., Feldott, G., and Lardy, H. A., *ibid.*, 1950, v73, 159.

16. Ames, S. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v166, 81.

17. Thompson, R. Q., and Guerrant, N. B., *J. Nutrition*, 1953, v50, 161.

18. Snell, E. E., *Physiol. Rev.*, 1953, v33, 509.

19. Dietrich, L. S., and Shapiro, D. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 555.

20. Lambooy, J. P., and Aposhian, H. V., *J. Nutrition*, 1952, v47, 539.

21. Snell, E. E., Klatt, O. A., Bruins, H. W., and Cravens, W. W., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 583.

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Changes in Serum Proteins Produced by Infusions of Dextran. (21939)

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(Introduced by David Seligson.)

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In the course of studies of possible metabolic effects of dextran infusions, a patient with chronic liver disease was given repeated infusions of dextran. A marked decrease in the concentration of serum proteins was observed, with a greater reduction in globulin than in albumin. Detailed studies were undertaken to confirm and evaluate these particular findings. For this purpose, 4 additional patients with liver disease and 8 patients convalescent from diseases other than liver disease were studied.

Methods. One patient was given only a single infusion of dextran, 750 cc of 12% dextran in water, the other 12 received daily infusions of 1000 cc of 6% dextran in saline. Five of these 12 men were given infusions for 4 successive days, 4 for 5 days, one for 6 days, and 2 for 15 days. Other than prolonged bleeding time(1), slightly prolonged one-stage prothrombin time, and minor headaches, no difficulties were encountered. Some patients gained as much as 3 kg of weight, but none developed overt edema.

Serum proteins were measured daily in most cases by the method of Reinhold(2), in which the globulins are precipitated by a sulfate-sulfite solution, and the protein in solution measured by the biuret reaction. On selected serums drawn both before and after dextran infusions the serum proteins were also checked by sulfate-sulfite precipitation and the analysis of supernatant nitrogen content by the Kjeldahl method. Filter paper electrophoresis was also used to check the partition of serum proteins, using the dye elution technique for colorimetric quantitation of the bromphenol blue bound by each protein zone. Dextran levels were measured by the turbidity method(3). In 3 of the liver disease patients serum cholinesterase(4), zinc turbidity (5), and thymol turbidity(6) were also measured.

Results. With repeated infusions of dextran, levels of dextran as high as 2.5 g % were found in the serum. Dextran was but slowly removed; appreciable levels were still present in the serum 10 to 15 days after infusion. Hematocrits and total serum protein concentrations changed inversely with the dextran level. In general the sum of the concentrations of protein and dextran remained constant, and roughly equalled the total pro-

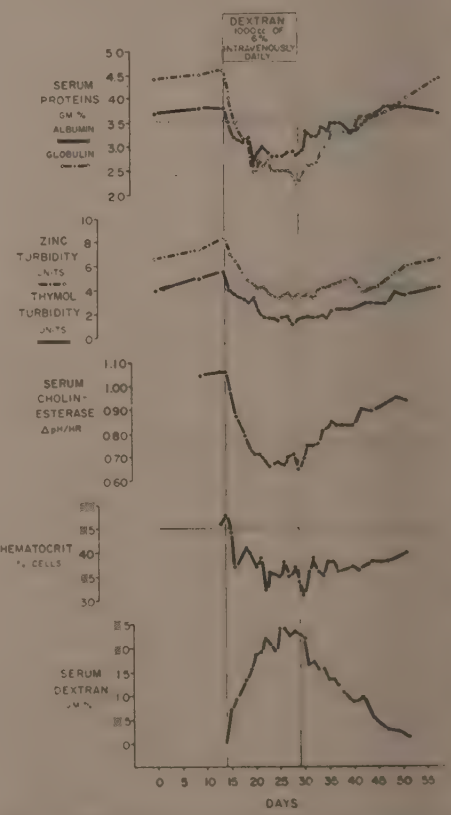

FIG. 1. Changes in serum proteins, hematocrit, serum dextran, serum cholinesterase, and zinc and thymol turbidity tests with repeated infusions of dextran in a patient with biliary cirrhosis convalescent from surgical relief of jaundice.

TABLE I. Correlations among Serum Proteins, Serum Dextran, and Hematocrit.

	Dextran, g %	Change in hematocrit, % cells
Change in total protein, g %	−.80	+.61*
" " albumin, "	−.77	+.44*
" " globulin, "	−.76	+.63*
" " A/G ratio	+.57	−.55
" " hematocrit, % cells	−.44	—

All correlations are highly significant. With 80 degrees of freedom (all correlations are based on 83 or more pairs) a correlation coefficient of .28 has a probability of .01.

* Correlation coefficients between percentage changes in serum proteins and hematocrit differ from those above by .03 or less.

tein concentration before dextran infusion.

In all patients, both liver disease patients and others, the serum globulin concentration decreased more than that of the serum albumin, so that the albumin:globulin ratios rose. These differential changes in albumin and globulin fractions were observed by both chemical methods used for analysis, and also in the paper electrophoresis patterns. By the latter technique the various globulins were found to be reduced equally.

In the patients in whom it was measured, the serum cholinesterase fell with dextran infusion. Abnormally high zinc and thymol turbidity values fell to the low normal range. (Fig. 1).

Discussion. Part of the reduction of serum protein concentration was certainly caused by expansion of the plasma volume by dextran. However, the increase in plasma volume calculated from changes in hematocrit accounted for only a part of the gain in body weight. Also it may be seen in Table I that the protein changes were less well correlated with the hematocrit changes than with the serum concentrations of dextran. The different percentage changes in albumin and globulin cannot be explained by plasma volume expansion. This difference was not caused by interference by dextran with the analytic method, because similar changes were found by the paper electrophoresis technique. Similar changes have been observed by Jaenike and Waterhouse(7).

Expansion of the interstitial fluid as well as the plasma volume must have occurred in these patients. Since the molecular size of the globulins is much greater than that of albumin the rate of transfer of globulins across the capillary wall should be slower than that of albumin. Therefore with transfer of fluid and protein from the plasma to interstitial fluid to compensate for the hypervolemia, the albumin concentration should fall faster than that of the globulin. A significant fall in total circulating albumin, but not in globulin, has been observed shortly after a rapid infusion of dextran(8). Later as dextran is metabolized and as fluid and dextran are excreted, albumin should return more rapidly to the plasma than globulin. This process of reequilibration may possibly explain the relatively greater reduction of globulin than of albumin which was observed.

The changes in albumin and globulin concentrations may also be explained by reductions in rates of synthesis. Since serum cholinesterase activity seems to parallel the rate of albumin synthesis(9), the observed reduction in cholinesterase may be taken as evidence of a decreased rate of albumin synthesis. Colloids similar to dextran are taken up by the reticulo-endothelial system, the site of globulin synthesis. Thus dextran might reasonably be expected to depress globulin synthesis. If dextran does reduce the rates of synthesis of albumin and globulin, there is no reason to expect, *a priori*, that the reductions need be the same.

The changes to normal of elevated zinc and thymol turbidity values are doubtless secondary to the relative changes in albumin and globulin.

Summary. Daily infusions of dextran were given to 13 patients for as long as 15 days. In addition to the decrease in total serum protein from plasma volume expansion, dextran was found to produce a relatively greater decrease in globulin than in albumin. Serum cholinesterase activity decreased, and zinc and thymol turbidity values fell.

1. Carbone, J. V., Furth, F. W., Scott, R., Jr., and Crosby, W. H., PROC. SOC. EXP. BIOL. AND MED., 1054, v85, 101.

2. Reinhold, J., Standard Methods of Clinical

Chemistry, Academic Press, Inc., New York, 1953, v1, 88.

3. Metcalf, W., and Rousselot, L. M., *J. Lab. Clin. Med.*, 1952, v40, 901.

4. Michel, H. O., *ibid.*, 1949, v34, 1564.

5. Kunkel, H. G., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 217.

6. Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, v162, 133.

7. Jaenike, J. R., and Waterhouse, C., *Circulation*, 1955, v11, 1.

8. Waldstein, S. S., Carbone, J. V., and Furth, F., to be published.

9. Vorhaus, L. J., Scudamore, H. H., and Kark, R. M., *Gastroenterology*, 1950, v15, 304.

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Effect of High Levels of Fat, Lactose, and Type of Bulk in Guinea Pig Diets.* (21940)

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Woolley and Sprince(1) reported that supplements of certain inert materials, such as powdered cellophane and Cellu flour, improved the growth of guinea pigs fed purified diets. Booth *et al.*(2) who found a similar beneficial action of bulk, reported gum acacia (formerly called gum arabic) to be slightly superior to Cellu flour. However, Reid and Briggs(3) could not always obtain consistent growth with diets containing gum acacia, and turned to the use of Spangles,† a coarsely-ground cellophane product.

In this paper we are presenting further studies on the effect of different types of bulk, especially Solka-floc,‡ a very pure, finely-ground and relatively inexpensive cellulose product, on the growth of guinea pigs as well as the effect of different levels of fat and lactose.

Methods. To test the effectiveness of these different materials, 3 types of diets were devised, including a "normal", a high-fat 24% corn oil, and one with lactose as the only carbohydrate. The latter 2 diets were chosen because more information was desired on the effect of these substances on the deposition of liver fat in guinea pigs fed milk diets(4). The basal or normal diet used in this experiment provided per 100 g of ration in g: sucrose, 43; casein, 30; bulk, 15; salts IV, 4(5); corn oil, 4; potassium acetate, 2.5; choline-Cl, 0.75; magnesium oxide, 0.50; and vitamin mix, 0.25. The vitamin mix provided per 100 g of ration in mg: inositol, 200; niacinamide, 20; p-aminobenzoic acid, 10; Ca-pantothenate, 8; riboflavin, 3; thiamine-HCl, 2; pyridoxine-HCl, 2; folic acid, 1; in μg: biotin, 100; B_{12}, 4. Fat soluble vitamins were provided weekly by dropper in the following amounts: A, 2000 I.U.; D, 20 I.U.; E, 12 mg; K, 0.2 mg. It should be noted that the diets contained 2.5 times the level of choline normally used in guinea pig diets to allow for any greater need with high-fat diets. The addition of corn oil to the high-fat diets and to those with graded levels of corn oil was made at the expense of sucrose. The care and feeding of guinea pigs have been described elsewhere(6). Since the diets differed so widely, the guinea pigs were initially divided into 3 groups with each group gradually being adapted to its particular diet. After adaptation, each of the 3 dietary groups was subdivided into 3 groups of 5 guinea pigs each, with the same average starting weight within each main group (normal, 194 g; high-fat, 221 g; and lactose, 217 g). The 3 normal groups were on experiment for 4 weeks after which they were redistributed into groups averaging 380 g. These groups were fed diets with 4, 8,

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† Rayon Processing Co., Pawtucket, R. I.

‡ Brown and Co., Berlin, N. H.

TABLE I. Comparison of Gum Acacia, Spangles and Solka-floc as Bulk for the Guinea Pig.

Diet and time	Avg growth in g/day		
	Gum acacia	Spangles	Solka-floc
Normal, 4 wk	6.5	6.1	7.4
High-fat, 6 wk	3.8	4.5	4.9
(% liver fat wet basis)	(5.1)	(5.4)	(4.3)
Lactose, 2 wk	1.0	1.8	2.0
Lactose, 4 more wk			2.6
½ lactose & ½ sucrose, 4 wk			5.6
Graded levels of corn oil	4%	8%	12%
(Solka-floc) 3 wk	5.8	5.3	7.7

and 12% levels of corn oil with Solka-floc as the bulk for 3 weeks. The 3 groups receiving high-fat diets were on experiment for 6 weeks, after which the livers were analyzed for fat by the method of Bixby *et al.*(7). The groups receiving lactose as the carbohydrate were on experiment for 2 weeks, after which the 12 remaining animals were redivided into 2 groups. One group containued to receive lactose as the only carbohydrate, and the other group had half of the lactose replaced by sucrose. Both groups received Solka-floc as the bulk, and were on experiment for 4 weeks.

Results and discussion. The results in Table I show that of the 3 types of bulk, Solka-floc supported slightly better growth with each type of diet. The feces from the guinea pigs receiving Solka-floc were the most normal in appearance. For these and other reasons previously mentioned, Solka-floc was used as the bulk in all subsequent guinea pig diets.

The general appearance of the guinea pigs receiving the high-fat diet with gum acacia, was definitely inferior to that of the other groups, probably due in part to the fact that gum acacia is less absorbent and left a more oily ration, which tended to stick to the fur of the animals. The livers of the animals receiving the high-fat diets were almost normal in appearance and fat in content. Although the livers of the group receiving Solka-floc had slightly less fat, none of the livers were very fatty since a normal guinea pig liver will average about 4% fat.

With high-fat diets, one animal died in the group receiving gum acacia and also one in the group receiving Spangles. However, the relatively good growth observed with the group receiving high-fat with Solka-floc was somewhat surprising. This suggested that the guinea pig could utilize high levels of corn oil. Consequently, the groups of animals that had been receiving the normal diets were redistributed and given diets with graded levels of corn oil. It is apparent from Table I that the group receiving 12% corn oil grew very well, somewhat better than the groups receiving 4 or 8% corn oil. As a result of this finding the corn oil content of all guinea pig diets for subsequent experiments was raised to 7.4% to permit easier mixing and to lessen the wastage of rations that become rather dry and loose with the use of Solka-floc and only 4% corn oil.

The guinea pigs receiving lactose as the sole carbohydrate grew very poorly, as can be seen by the growth of only 2.0 g or less/day. One animal from each group died within the first 2 weeks. When the remaining animals were redivided into 2 groups, the substitution of sucrose for ½ the lactose in this diet resulted in a marked improvement in growth. This group gained an average of 5.6 g/day for 4 weeks as compared to the growth of only 2.6 g/day for the group continuing to receive lactose as the only carbohydrate. This would indicate that there is very little adaptation by the guinea pig to utilize lactose under these conditions.

The results of these experiments would also suggest that the poor growth of guinea pigs fed milk diets is primarily due to the lactose in the diets, although preliminary evidence(4) indicates that these diets may also be deficient in potassium, magnesium, arginine, and possibly other substances. Although these experiments do show that guinea pigs can utilize 12% corn oil very effectively and 24% rather well, they do not shed much light on the cause of the very fatty livers found in guinea pigs fed milk diets. The possibilities still exist that deficiencies in vitamins or amino acids or differences in kind and level of fat may be etiological factors in this condition.

Summary. Gum acacia, Spangles, and Solka-floc have been compared as bulk agents

for the guinea pig by employing normal, high-fat, and lactose diets. The average growth rate of the groups receiving Solka-floc was slightly better in all cases. Guinea pigs grew very well on diets containing 12% corn oil (7.7 g/day) and at a rate of 4.9 g/day with 24% corn oil diets using Solka-floc as the bulk. Guinea pigs fed diets with lactose as the sole carbohydrate grew at a rate of about 2.0 g/day. Substitution of sucrose for half of the lactose increased growth to over twice the rate obtained with lactose alone.

1. Woolley, D. W., and Sprince, H., *J. Biol. Chem.*, 1945, v157, 447.

2. Booth, A. N., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1948, v37, 263.

3. Reid, M. E., and Briggs, G. M., *ibid.*, 1953, v51, 341.

4. Heinicke, H. R., and Elvehjem, C. A., unpublished data.

5. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

6. Heinicke, H. R., Harper, A. E., and Elvehjem, C. A., *J. Nutrition*, in press.

7. Bixby, J. N., Bosch, A. J., Elvehjem, C. A., and Swanson, A. M., *Ag. and Food Chem.*, 1954, v2, 375.

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Hydrolysis of Succinylmonocholine by a Liver Esterase.* (21941)

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Succinyldicholine (SDC), a short-acting muscle relaxant(1), has replaced to a considerable extent the older relaxants d-tubocurarine and decamethonium since its introduction in 1949(2). Little systematic work, however, has yet been reported on its mode of breakdown in the body. It is known(3) that there is little excretion of unchanged SDC after intravenous injection and that, *in vitro*, serum cholinesterase hydrolyses SDC to succinylmonocholine (SMC) and choline(4,5). This enzyme also hydrolyses SMC to succinic acid and choline, but at a much lower rate(5,6). In many patients with a low serum-cholinesterase level, a prolonged response to SDC is encountered(7,8) but it is also known that a similar abnormal prolongation of the period of relaxation after SDC administration can occur in subjects with normal serum-cholinesterase levels(9,10). It seems possible that this lack of correlation between serum-cholinesterase level and duration of relaxation may be due to the presence of another esterase which is also capable of hydrolysing SDC. It has also been suggested(11) that SMC, which itself has slight muscle-relaxant properties (12), accumulates in the body after SDC administration and potentiates the action of SDC. Since the hydrolysis of SMC by serum cholinesterase is slow, especially in the presence of SDC(5), SMC might be expected to accumulate under these conditions unless there is another esterase in the body which is also capable of hydrolysing SMC.

It was therefore decided to determine whether esterases hydrolysing SDC or SMC are present in other tissues. The present communication concerns such an enzyme which is present in liver and which is distinct from liver cholinesterase. This enzyme catalyzes the hydrolysis of SMC but is without action on SDC.

Materials and methods. Samples of succinyldicholine dichloride and succinylmonocholine iodide were kindly supplied by Burroughs-Wellcome & Co. The rats used were a local hooded strain (170-220 g). The animals were stunned and exsanguinated, and the tissues removed and placed in cracked ice. Homogenates (17% in water) were prepared in Potter-Elvehjem type homogenizers at 0-5°C. The rates of enzymatic and non-enzymatic ester hydrolysis were followed in

* We are most grateful to Messrs. Burroughs-Wellcome and Co., Inc., Tuckahoe, N. Y. for a grant which made this work possible.

TABLE I. Hydrolysis of SMC and SDC by Rat Liver Homogenate.

Substrate	Rate of hydrolysis, μl CO_2/hr above endogenous		
	Without enzyme	With 170 mg homogenized rat liver	With 340 mg homogenized rat liver
.01*M* SMC	0	55	97
.02*M* "	1	54	110
.01*M* SDC	14	33	29
.02*M* "	28	58	58

the conventional Warburg apparatus by measurement of the CO_2 evolution from a 0.025 *M* sodium bicarbonate medium under anaerobic conditions at 37°C. The initial concentration of ester was normally 0.01 *M*, the total volume 3.0 ml and the vessels were gassed with 93% N_2-7% CO_2 for 20 minutes. The substrates were tipped in from the sidearms and after a further period of 10 minutes an initial reading was taken. Readings were then taken every 10 minutes for a period of one hour. Liver homogenate was separated into sub-cellular fractions according to the method of Schneider (13).

Results. Rat liver homogenate increases the rate of production of CO_2 from SDC and, to a greater extent, from SMC. Unlike the hydrolysis by serum, this liver-catalysed hydrolysis was not inhibited by 10^{-3} *M* eserine or by 10^{-4} tetraethylpyrophosphate (TEPP). However, a study of the kinetics of SDC hydrolysis showed that the rate of hydrolysis was not increased when the concentration of liver homogenate was doubled, but that the rate was doubled by doubling the concentration of SDC (from 0.01 *M* to 0.02 *M*). With SMC, however, doubling the enzyme concentration doubled the rate of hydrolysis but increasing the substrate concentration from 0.01 *M* to 0.02 *M* gave no increase in activity. Typical results are given in Table I. Since SDC has an appreciable rate of non-enzymatic hydrolysis under the given experimental conditions, the enzymatic hydrolysis of the SMC produced by this process would account for the observed activity in the SDC-liver homogenate system. These observations led to the conclusion that the liver esterase hydrolyses SMC but not SDC. The enzyme is provisionally termed "succinylmonocholine esterase" (SMC-esterase). The SMC-hydrolysing activities of rat, guinea pig, rabbit and human liver are shown in Table II.

Distribution of enzyme activity in rat liver. A 30% homogenate of rat liver in 0.25 *M* sucrose was fractionated by differential centrifugation at 0°C into nuclear, mitochondrial, microsomal and soluble fractions. As shown in Table III, most of the SMC-hydrolysing activity is located in the mitochondrial fraction. The slight activity observed in the nuclear fraction is probably due to the presence of some unbroken cells. In preliminary work on SMC-esterase, suspensions of guinea pig and rat liver mitochondria, prepared by fractional centrifugation in 0.25 *M* sucrose, were used as a source of the esterase. However, owing to the relative instability of these preparations, an attempt was made to prepare a stock of semi-purified enzyme in concentrated solution.

Preparation of a purified SMC-esterase. Acetone-dried rat liver was prepared by homogenizing rat liver with ice-cold acetone in a Waring blender, filtering by suction and washing several times on the filter with cold acetone. The powder was dried and stored in a vacuum desiccator at 0°C. The resulting powder contained an active SMC-esterase, but the enzyme could not be extracted from the powder with water. A suitable extraction medium was found to be 0.25 *M* ammonium

TABLE II. Hydrolysis of SMC by Liver Tissues.

Enzyme preparation	Rate of hydrolysis of 0.01*M* SMC, μl CO_2/hr/100 mg liver
Rat liver homogenate	30, 31, 33, 36
" " slices	34
Guinea pig liver homogenate	26, 26, 27, 28, 34
Rabbit liver homogenate	7, 8
Human liver homogenate (36 hr post-mortem)	13
Human liver homogenate* (fresh)	7

* This sample was removed as a small slice (250 mg) during a cholecystectomy operation and immediately frozen in an acetone-solid CO_2 mixture. Enzyme activity was determined 1 hr later. The low value may be due either to freezing procedure employed or to liver damage in the patient.

TABLE III. Hydrolysis of SMC by Cellular Fractions of Rat Liver.

Fraction	SMC-hydrolysing activity, μl CO_2/hr/mg nitrogen*
Whole homogenate	10
Nuclear fraction	15
Mitochondria	130
Microsomes	5
Soluble	1

* Determined by micro-Kjeldahl method.

hydroxide. A 5% suspension of the rat liver powder in this medium was homogenized for 2 minutes in a Waring blender in the presence of a few drops of octyl alcohol. The suspension was allowed to stand at 5°C for 15 minutes and then centrifuged at 20,000 x gravity for 15 minutes. The viscous supernatant was brought to pH 7.4 with 5% acetic acid and the heavy precipitate formed was removed by centrifugation at 3,300 x gravity for 10 minutes. The resulting supernatant contained 60% of the total activity present in the original acetone powder. The extract was then fractionated with ammonium sulfate. The precipitate formed at 33% saturation with ammonium sulfate had only slight esterase activity with SMC as substrate, but when the concentration of ammonium sulfate was raised to 50% saturation, a precipitate was formed which contained 60% of the activity of the unfractionated extract. The supernatant from this precipitate contained no detectable activity. The activity of all fractions was determined after dialysis against running tap-water overnight.

The fraction with the high SMC-esterase activity (33-50% saturation) had a very low cholinesterase activity with acetyl- or benzoylcholine as substrate. Further, the SMC-esterase was unaffected by 10^{-4} *M* TEPP, whereas the cholinesterase was completely inhibited. Work is now in progress to determine the substrate specificity of the purified preparation of SMC-esterase and to study its resistance to other esterase inhibitors.

Discussion. SMC-esterase might prevent the accumulation of SMC in the body after SDC administration if present in an active form in man. This would suggest that SMC accumulation is not responsible for the prolonged effect of SDC sometimes observed. However, it is possible that in such cases the liver SMC-esterase level and not the serum cholinesterase level is abnormally low and thus allows the accumulation of SMC. It is of interest that a choline ester, namely SMC, should be hydrolyzed in liver by an enzyme which is distinct from the cholinesterases of Whittaker's classification(14).

Summary. 1. The livers of rat and of other animals, including man, contain an esterase, distinct from known cholinesterases, which hydrolyses succinylmonocholine. The enzyme is located in the mitochondria and can be extracted from a liver acetone-powder with dilute ammonium hydroxide. It has been partially purified by ammonium sulfate fractionation of a liver extract, and the final preparation was almost free of cholinesterase activity. The enzyme is TEPP insensitive. 2. The possible role of this enzyme in recovery of muscular control after succinyldicholine administration is discussed.

1. Bourne, J. G., Collier, H. O. J., and Somers, G. F., *Lancet*, 1952, v1, 1225.

2. Bovet, D., Bovet-Nitti, F., Guarino, S., Longo, V. G., and Marotta, M., *Rendiconti Ist. Sup. Sanita*, 1949, v12, 106.

3. Foldes, F. F., Vandervort, R. S., and Shanor, S. P., *Anesthesiology*, 1955, v16, 11.

4. Tsuji, F. J., and Foldes, F. F., *Fed. Proc.*, 1953, v12, 374.

5. Whittaker, V. P., and Wijesundera, S., *Biochem. J.*, 1952, v52, 475.

6. Foldes, F. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 187.

7. Evans, F. T., Gray, P. W. S., Lehmann, H., and Silk, E., *Lancet*, 1952, v1, 1229.

8. Forbat, A., Lehmann, H., and Silk, E., *ibid.*, 1953, v1, 1067.

9. Borders, R. W., Stephen, C. R., Nowill, W. K., and Martin, R., *Anesthesiology*, 1955, v16, 401.

10. Hodges, R. H. J., and Harkness, J., *Brit. Med. J.*, 1954, v2, 18.

11. Foldes, F. F., McNall, P. G., and Birch, J. H., *ibid.*, 1954, v1, 967.

12. Ellis, C. H., Wnuck, A. L., Fanelli, R. V., and de Beer, E. J., *J. Pharmacol.*, 1953, v109, 83.

13. Schneider, W. C., *J. Biol. Chem.*, 1948, v176, 259.

14. Whittaker, V. P., *Physiol. Rev.*, 1951, v31, 312.

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Alterations in Renal Threshold for Sugar by Oral Administration of Cortisone Acetate. (21942)

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Because of widespread clinical use of ACTH and cortisone, their influence upon renal threshold for sugar is of more than academic interest. Holten and Lundbraek(1) noted glycosuria without appreciable rise in fasting or maximum blood sugar levels in well controlled patients by administration of 100 mg of ACTH daily. These findings were confirmed by Kass(2), whereas Fillipis(3), working independently, found the renal threshold for glucose to be significantly increased after use of ACTH in single doses not higher than 40 mg. The effect of DOC glucoside on renal tubular reabsorption in dogs was investigated by Despopoulos and Kaufman(4), who found a reversible depression of renal reabsorption capacity for glucose. To obtain such data after the use of cortisone, renal threshold determinations were made in 8 patients.

Methods and procedures. Four patients were studied, 2 men and 2 women, aged 75, 77, 59, and 64, respectively. One had a diaphragmatic hernia and no other pathology. The other 3 were objects of custodial care without any obvious pathologic conditions, except a degree of arteriosclerosis compatible with age. None of the patients had ever had evidence of diabetes mellitus. After 10 P.M. of the evening preceding each test, food and fluids were withheld. An indwelling catheter was inserted 15 minutes before the test and the bladder emptied. After a fasting blood sugar specimen had been taken, infusion of a 20% solution of glucose in water was started. The infusion was allowed to flow at a speed of 10 ml per minute. The blood and urine were examined for glucose at 5-minute intervals, the infusion stopped as soon as a positive reaction for sugar was found in the urine. For each of 6 immediately consecutive days, the patients were given 200 mg of cortisone acetate orally and their regular ward diets were kept at an isocaloric level of 2200 calories. On the 7th day, the above procedure was repeated. Before, during and after the test, patients were on a regular ward diet. This method provided us with some rather strikingly uniform data about the "breakdown" of the kidney threshold, as a result of administration of glucose and glucose in conjunction with cortisone acetate. In a second group of 4 patients, we determined not only the point at which the renal threshold was exceeded, but also the point of its restoration after treatment with glucose alone and glucose plus cortisone acetate, respectively. The technic remained essentially the same as for the first study, except that values for glucose in blood and urine were determined after stopping the infusion until the urine was found to be aglycosuric. The subjects of the second group, 2 of whom were men, 69 and 76 years old, respectively, and 2, women, 70 and 72 years old, respectively, were free of any active organic disease, other than the vascular changes compatible with age.

Results. Study I. In all 4 patients of the first group, we found a lowered threshold for glucose following treatment with cortisone. Glycosuria occurred at definitely lower level for blood sugar. The difference between values before and after cortisone varied from 52 to 132 mg per 100 ml (Table I and Fig. 1). The average decrease in the threshold for glucose was 86 mg per 100 ml. Administration of cortisone was associated with a shortening of the time necessary to exceed the renal threshold for glucose in 2 subjects, a lengthening in one, and no change in the fourth (Table I).

Study II. In the second group of 4 patients, we confirmed the findings of Study I, although in case No. 7 (Table I) the decrease in the threshold was not as evident as in the other subjects. In this individual, during the first test, glucose appeared in the urine between

* Wakefield Foundation Fellow in Research.

TABLE I. Influence of Cortisone on Renal Threshold for Glucose in 8 Normally Aging Subjects. Determinations of glucose in blood (mg/100 cc) and urine at periodic intervals (min.).

Pt. No.	Sex	Age (yr)		Before and after treatment with cortisone — Time in min. 0 B*	0 A	5 B	5 A	10 B	10 A	15 B	15 A	20 B	20 A	25 B	25 A	30 A	45 B	45 A	50 B	50 A	55 B	55 A	60 B	60 A	65 B	65 A	70 B	70 A	75 A
1	♀	75	Blood	98	120	206	253	240	364	416	478	560	544	630															
			Urine	0	0	0	0	0	1+	1+	2+	2+	3+	3+															
2	♀	77	Blood	101	89	236	253	369	305	437	358	518	415																
			Urine	0	0	0	0	0	3+	3+	3+	4+	4+																
3	♂	59	Blood	142	115	229	183	370	248	434	287	443	327	495	334	438													
			Urine	0	0	0	0	0	0	0	0	2+	0	3+	1+	4+													
4	♂	64	Blood	99	66	144	152	234	166	282	220	344	290	368	302														
			Urine	0	0	0	0	0	0	1+	1+	3+	3+	4+	4+														
5	♂	69	Blood	102	100	205	187	306	322	347	330	395	372						221	194	205	188		170					
			Urine	0	0	0	0	0	0	2+	2+	4+	4+						2+	1+	0	1+		0					
6	♂	76	Blood	121	99	334	281	501	346	532		451						362		302		283	342					245	179
			Urine	0		0	—	2+	1+	3+		4+						2+		3+		3+	1+					0	0
7	♀	70	Blood	70	91	220	218	327	358	346							299	235	272	192	251	185	222	160	217				
			Urine	0	0	0	0	0	3+	3+							4+	3+	4+	2+	4+	2+	1+	0	0				
8	♀	72	Blood	122	108	154	172	196	311	345	370	428	403	512				380	482	338	396	274	278	224		162			
			Urine	0	0	0	0	0	1+	2+	3+	3+	4+	4+				4+	4+	3+	2+	3+	0	1+		0			

* B = Before; A = After.
Solid underline shows point of break in tolerance. Dotted underline shows point of restoration of tolerance.

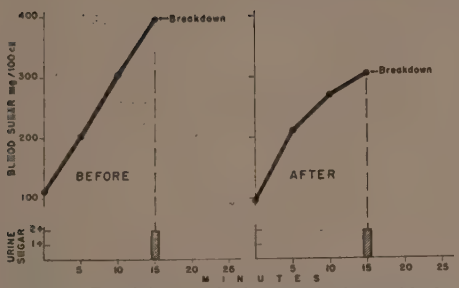

FIG. 1. Breakdown of the renal threshold for sugar before and after treatment with cortisone acetate—200 mg orally daily for 6 days (arithmetical averages for cases 1-4).

FIG. 2. Breakdown and restoration of the renal threshold for sugar before and after treatment with cortisone acetate—200 mg orally daily for 6 days (arithmetical averages for cases 5-8).

blood sugar levels of 327 and 346 mg per 100 ml, while after administration of cortisone acetate this occurred between 218 and 358 mg per ml. During the 5-minute span, it seems likely that glucose may have been excreted into the bladder before the higher level of 358 mg per 100 ml was reached.

In addition to confirmation of the previous findings, we determined the "restoration" of the kidney threshold in these 4 subjects. In all of them, there was a marked decrease in the blood sugar level at which restoration of the threshold occurred following the use of cortisone acetate (Table I and Fig. 2). The differences before and after the use of cortisone acetate varied from 35 to 116 mg per 100 ml with an average of 89 mg per 100 ml. Not only was the renal threshold for sugar lowered by cortisone, but also all values for blood sugar seemed to be depressed, thus producing a depressed or low glucose tolerance curve (Fig. 2). Following administration of cortisone acetate, the time necessary to exceed the renal threshold for sugar was unchanged in 2 and shortened in 2 (Table I). The time required to "restore" the renal threshold for sugar was not altered by the amounts of cortisone given in these studies (Table I and Fig. 2).

Summary. A simple method is described for determination of the renal threshold for glucose in untreated and cortisone-treated individuals. In 4 patients the "breakdown" of the glucose-threshold was investigated and was found to be markedly decreased after 6 days of orally administered cortisone, 200 mg daily. The decrease amounted to an average of 86 mg per 100 ml of blood with a range from 52 to 132 mg per 100 ml. In another group of 4 patients, both the "breakdown" and the "restoration" thresholds were determined. The breakdown values for the first group were confirmed and in addition the restoration of the threshold was also found to be at a lower level for blood sugar, averaging 68 mg per 100 ml less after cortisone than before it with individual variations from 35 to 116 mg per 100 ml.

1. Holten, C., and Lundbraek, K., *Scandinav. Clin. and Lab. Invest.*, 1950, v2, 317.

2. De Filippis, V., *Lancet*, 1951, v1, 856.

3. Kass, E. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 669.

4. Despopoulos, A., and Kaufman, E. H., *Am. J. Physiol.*, 1952, v170, 11.

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Suppression of Adrenal Compensatory Hypertrophy by Hypothalamic Lesions.* (21943)

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There is general agreement that lesions of the median eminence suppress the acute adrenal cortical response to stress(1,2,3). Interruption of the supraopticohypophyseal tract as evidenced by diabetes insipidus appears to be responsible for this failure of the normal stress-induced discharge of adrenocorticotrophin (ACTH)(4). In the adrenalectomized rat maintained on desoxycorticosterone, acute stress results in very high levels of blood ACTH. If rats with hypothalamic lesions are adrenalectomized and subjected to acute stress, no detectable ACTH can be found in blood(5). In addition to acute stress, it is known that adrenalectomy results in an increase in pituitary ACTH output(6,7). This increase can not be demonstrated in adrenalectomized rats with hypothalamic lesions, and this suggests that the increased ACTH secretion following adrenalectomy may also be dependent upon hypothalamic activity(5). However, small increases in blood ACTH concentration above the normal, unstressed level would not be detected by the assay employed. It was of interest, therefore, to determine whether the compensatory hypertrophy which follows unilateral adrenalectomy would occur in rats with hypothalamic lesions. This hypertrophy is presumably due also to increased ACTH discharge consequent to a fall in blood steroids following unilateral adrenalectomy(8,9). The present results indicate that lesions which interrupt the supraopticohypophyseal tract suppress the compensatory hypertrophy following unilateral adrenalectomy, but they also suggest that a small response may be present.

Methods. Bilateral lesions were produced in the hypothalamus of adult male rats of the Wistar strain with the aid of a Krieg stereotaxic instrument. Three weeks to 2 months postoperatively, the rats were subjected to left unilateral adrenalectomy under ether anesthesia. Two weeks later the right adrenal was removed. Control rats without lesions were similarly studied. Adrenals were weighed to the nearest 0.1 mg on a torsion balance. Because of variations in initial adrenal weights, all results are expressed as the percentage change in right adrenal weight after unilateral adrenalectomy as compared to the weight of the previously removed left adrenal.

Results. Twelve normal rats subjected to unilateral left adrenalectomy exhibited an hypertrophy of the right adrenal equal to 51 ± 8%, when it was removed 2 weeks later. Since previous work has shown that the blockade in the acute adrenal stress response in rats with lesions correlates well with the degree of interruption of the supraopticohypophyseal tract as judged by increased water intake(4), the compensatory adrenal hypertrophy of the control rats and of 48 rats with lesions of the hypothalamus was plotted against their daily water intake in Fig. 1. It

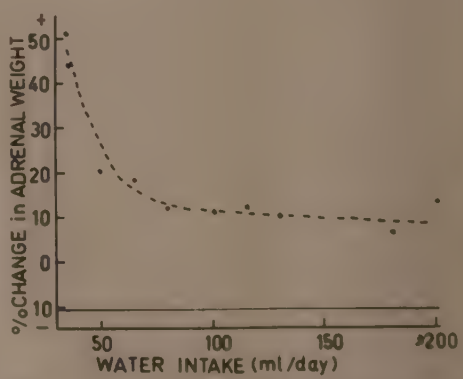

FIG. 1. Suppression of compensatory adrenal hypertrophy in rats with diabetes insipidus. Daily water intake on abscissa is plotted against percentage change in right adrenal wt. Each point represents mean response of 4 or more rats for each increment of 10 ml or a multiple thereof of water consumption per day. Solid line represents result found in rats with lesions where both adrenals were removed simultaneously.

* Supported by a grant to John R. Brobeck from the National Science Foundation.

can be seen that the degree of hypertrophy fell off very rapidly as water intake increased above the normal level of 35 ml/day. At levels of water intake above 100 ml/day, the compensatory hypertrophy fell to a minimal value of approximately 10%, and it remained at this figure with increasing values of water intake. The mean increase in right adrenal weight of all 20 animals with water intakes of 100 ml/day or more was $+9 \pm 4\%$. In another series of rats with hypothalamic lesions, when the 2 adrenals were removed simultaneously, the right adrenal weighed on the average $11 \pm 2\%$ less than the left adrenal. Consequently, in the rats with diabetes insipidus the response to unilateral adrenalectomy was $+9 \pm 4\%$ plus $11 \pm 2\%$, or a total gain of approximately 20% above the expected weight of glands from this location in rats with lesions. This gain is 30% of the gain exhibited by normal animals. This small residual response was statistically significant if compared to the results obtained when both adrenals were removed simultaneously in the rats with lesions ($P<0.01$).

Nearly all rats gained weight during the experimental period. Five of the 53 rats with lesions, however, lost 10% or more of their body weight during this time, and all of these 5 animals exhibited hypertrophy of the remaining adrenal, the mean response being $+56 \pm 14\%$, a value similar to that of the control rats without lesions. This hypertrophy in rats with weight loss was independent of the water intake which varied between 90 and 380 ml/day. The results in these rats were excluded from consideration in Fig. 1.

Testicular atrophy occurred in only 6 of the 52 rats studied, a result in harmony with previous studies indicating that suppression of adrenal function can occur in the absence of testicular atrophy(1,5).

Discussion. The present results indicate that compensatory adrenal hypertrophy is suppressed in rats with hypothalamic lesions. This is interpreted to mean that such lesions at least partially block the augmented secretion of pituitary ACTH which normally ensues when the blood level of corticoids is lowered as a result of unilateral adrenalectomy. A small response still occurs in such rats. Furthermore, if the animals lose significant weight during the course of the study, they still manifest compensatory hypertrophy. This hypertrophy is presumed to be due to some additional stress which appears to be capable of evoking a discharge of ACTH when acting over this 2-week time interval even in the rat with hypothalamic lesions. It has been previously demonstrated that rats with extremely severe diabetes insipidus show adrenal hypertrophy although the acute adrenal stress response is blocked(4,5). Sufficient rats with severe diabetes insipidus have not been subjected to unilateral adrenalectomy to allow conclusions as to whether they would still respond with compensatory hypertrophy.

The lesions in the present study were designed to interrupt the supraopticohypophyseal tract in the rostral portion of the median eminence. Damage to other structures undoubtedly occurred, but the correlation between diabetes insipidus and reduction in the response to unilateral adrenalectomy agrees with the results of previous work in which it was demonstrated that blockade of the acute adrenal stress response in rats could be correlated only with injury to the supraopticohypophyseal tract(4).

Our results are in good agreement with those recently reported by Ganong and Hume in the dog(10), who correlated a complete absence of compensatory hypertrophy with lesions which destroyed an estimated 50% or more of the median eminence. In our experience a small response is still present in rats with lesions.

The secretion of ACTH which normally follows adrenalectomy is believed to be caused by the fall in blood level of corticoids following removal of the adrenals(5,7). In animals with appropriate hypothalamic lesions there is a suppression of this ACTH secretion. It appears that this suppression may have two possible explanations. Either the steroids normally act directly upon the hypothalamic or other nervous structures to inhibit neural activity when the blood level is raised and to stimulate it when the level falls. Or if the steroids act directly upon the cells of the anterior pituitary gland, then the sensitivity of

these cells to the level of cortical steroids present in the blood must be affected by the neurohumor presumed to be secreted by the hypothalamus. Our data do not permit us to decide which of these possibilities is correct.

Conclusions. The compensatory adrenal hypertrophy which normally follows unilateral adrenalectomy is suppressed in rats with diabetes insipidus produced by hypothalamic lesions.

1. McCann, S. M., *Am. J. Physiol.*, 1952, v171, 746.
2. Hume, D. M., *Ann. Surg.*, 1953, v138, 548.
3. Anand, B. K., Raghunath, P., Dua, S., and Mohindra, S., *Indian J. Med. Research*, 1954, v42, 2.
4. McCann, S. M., and Brobeck, J. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 318.
5. McCann, S. M., and Sydnor, K. L., *ibid.*, 1954, v87, 369.
6. Gemzell, C. A., Van Dyke, D. C., Tobias, C. A., and Evans, H. M., *Endocrinol.*, 1951, v49, 325.
7. Sydnor, K. L., and Sayers, G., *ibid.*, 1954, v55, 621.
8. Tepperman, J., Engel, F. L., and Long, C. N. H., *ibid.*, 1943, v32, 373.
9. Ganong, W. F., *ibid.*, 1954, v55, 117.
10. Ganong, W. F., and Hume, D. M., *ibid.*, 1954, v55, 474.

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Plaque Formation of Poliomyelitis Viruses on Human Amnion Cell Cultures.* (21944)

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In a previous publication(1) human amnion cells were reported to support propagation of poliomyelitis viruses, and it was indicated that tissue cultures obtained from this readily available tissue could provide a source for the large scale production of poliomyelitis virus.

In the present work plaque formation in monolayer tissue cultures of amnion cells by the 3 types of this virus was obtained, similar to the plaque formation by this virus in monkey kidney and testis cultures(2) and human cancer (HeLa) cell cultures(3). It is, therefore, possible to employ this tissue also for the quantitative assay of poliomyelitis virus.

Material and methods. Virus. Poliomyelitis viruses, type 1, strain Mahoney; type 2, strain MEF-1; type 3, strain Saukett, obtained from Dr. Jonas Salk in 1952, were passed several times in monkey kidney cells, 3-4 times in HeLa cells and finally 1 or 2 times in monkey kidney cells. The final virus preparations were stored at –60°C. *Cells.* Monolayer cultures of human amnion cells were prepared in 50 mm petri dishes as previously described(1). After centrifugation the cell pack was diluted 1:120 and the cultures were grown at 37°C in a 3% CO_2-atmosphere in a medium consisting of 20% ox serum and Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate (Nutritional Biochemicals). The culture fluid was replaced by fresh medium after incubation for 4-5 days. The cell sheet was usually confluent after 10-14 days. *Infection and overlay.* The cultures were washed 3 times with buffer solution at 37°C after removal of the nutrient fluid and inoculated with 0.3 ml of various dilutions of the virus preparation. After permitting adsorption of the virus for at least 30 minutes at 37°C, the plates were overlayed with 3-4 ml of melted agar containing nutrient fluid. 100 ml nutrient fluid consisted of 55 ml 1% Difco yeast extract in distilled water, 6 ml 5% bovine albumin powder V (Armour) in Hanks' solution, 9 ml 5% $NaHCO_3$ in distilled water and 30 ml 10 times concentrated Hanks' solution without phenol red(4). One part of 3% agar was melted and kept in a water bath at 44°C. One part of the nutrient fluid was mixed with an equal volume of dis-

* Aided by a grant from the American Cancer Society and a grant from the National Foundation for Infantile Paralysis.

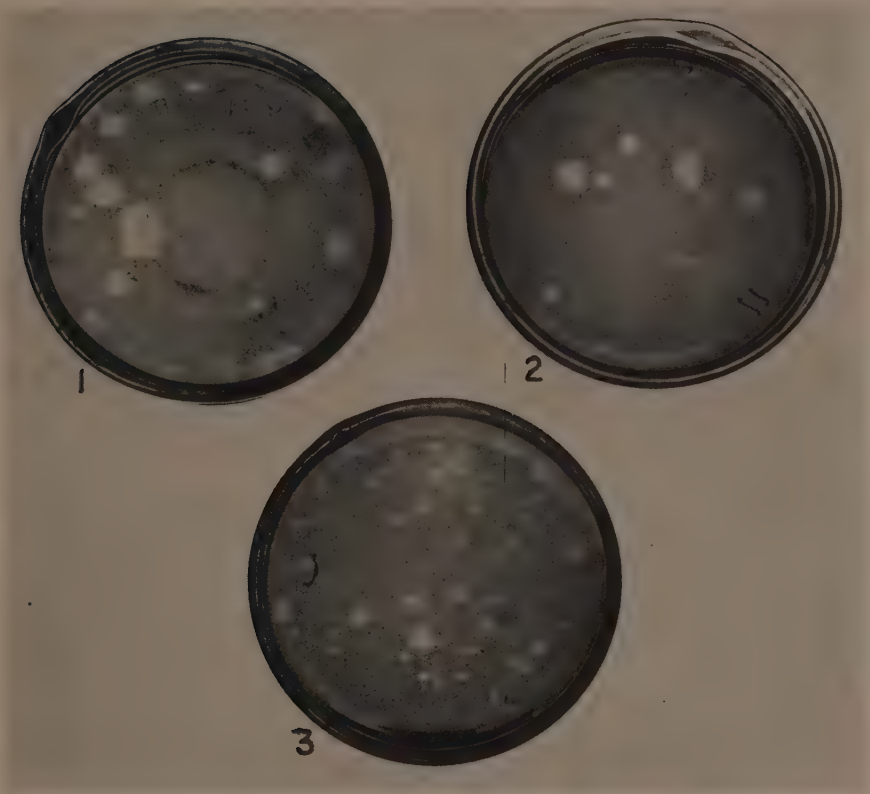

FIG. 1. Plaques of poliomyelitis viruses on human amnion cell cultures 72 hr after infection. 1: Type 1, strain Mahoney; 2: Type 2, strain MEF-1; 3: Type 3, strain Saukett.

tilled water and added to the agar. After 2½ days of incubation at 37°C the plates were stained with 0.3 ml of 1:4000 neutral red in phosphate buffer solution.

The monkey kidney plate cultures used for comparative assays were prepared by the technique devised by Dulbecco and Vogt(*2*) and grown in a medium consisting of 2% ox serum and Earle's balanced salt solution with 0.5% lactalbumin hydrolysate. The plates were infected, overlayed and incubated as were the amnion plates.

Results. Fig. 1 shows the plaques produced by inoculating suitable dilutions of the 3 types of poliomyelitis virus on monolayer cultures of first generation human amniotic cells. The plaques were almost round areas which could be easily seen and counted without magnification. The size of the plaques was somewhat variable and increased with time of incubation until the plaques became confluent. At an early time when the plaques were still small it was possible to count up to approximately 200 plaques in one plate, since they were very easily discernible.

Table I shows a comparison of titers obtained for 4 different samples assayed on monkey kidney and human amnion plate cultures, at the same time under identical conditions. It can be seen that the number of plaque forming units per ml of Mahoney and MEF-1 virus estimated from the amnion plates was consistently higher than that from the monkey kidney plates. This difference was not observed for the single Saukett virus assay. More detailed comparison of the titers obtained in the 2 tissue culture systems is being made in this laboratory.

TABLE I. Number of Plaques and Calculated Plaque Forming Units (PFU) per ml for 4 Samples of Poliomyelitis Virus Assayed on Monkey Kidney and Human Amnion Plates in Various Dilutions.

Virus samples	Dilution	Cell layer from: Monkey kidney Plaques/plate	Monkey kidney PFU/ml	Human amnion Plaques/plate	Human amnion PFU/ml
Mahoney	$10^{-5.5}$	19, 20, 21, 19	2.1×10^7		
	10^{-6}	9, 7, 5, 5	2.2×10^7	25, 24	8.2×10^7
	10^{-7}			0, 6	1.0×10^5
"	10^{-6}	38, 41, 43	1.4×10^8		
	$10^{-6.5}$	24, 17, 15	2.0×10^8		
	10^{-7}	2, 5, 4	1.2×10^8	26, 26	8.7×10^8
	$10^{-7.5}$			8, 11, 11	1.1×10^6
MEF-1	10^{-6}	25, 25, 34	8.9×10^7	113, 115	3.8×10^8
	$10^{-6.5}$	10, 10, 7	9.6×10^7		
	10^{-7}	2, 4, 3	1.0×10^8	10, 4	2.3×10^8
Saukett	10^{-4}			217, 189	6.8×10^6
	10^{-5}	88, 107, 90	3.2×10^7	85, 68	2.6×10^7
	$10^{-5.5}$	31, 32, 31	3.3×10^7		
	10^{-6}	3, 11	2.3×10^7	3, 5	1.3×10^7

Summary. Plaque formation of all 3 types of poliomyelitis virus has been obtained in monolayer cultures of human amnion cells. The plaque titer obtained on amnion was 3-6 times higher than on monkey kidney cell cultures for strains Mahoney and MEF-1.

1. Zitcer, Elsa M., Fogh, J., and Dunnebacke, Thelma H., *Science*, 1955, v122, 30.

2. Dulbecco, R., and Vogt, Marguerite, *J. Exp. Med.*, 1954, v99, 167.

3. McClain, Mary E., and Schwerdt, C. E., *Fed. Proc.*, 1954, v15, 505, abstract.

4. Youngner, J. S., 1955, personal communication.

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Propagation of Newcastle Disease Virus in Ehrlich Ascites Cells *In vitro* and *In vivo*.* (21945)

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(Introduced by Herald R. Cox.)

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Mengo encephalitis virus has been cultivated in the Ehrlich ascites carcinoma cell *in vitro*(1). While no cytopathogenic effect of Mengo virus—in spite of its multiplication—was visible in the test tube, its oncolytic property was readily demonstrated in the ascites cells *in vivo*. Some workers have reported the inhibition of Ehrlich ascites tumor growth when the inoculum was first mixed with Newcastle disease virus (NDV) either just prior to or at the time of inoculation(2). However, sustained multiplication of the NDV in the Ehrlich cells could not be demonstrated(3).

It seemed of interest to ascertain whether this virus could be adapted to grow in the Ehrlich ascites tumor and to determine whether viral multiplication would result in oncolysis *in vivo*.

Material and methods. Virus. The Newcastle disease virus was originally isolated in Massachusetts in 1945 by Dr. Van Roekel, and is designated MD20Z. It was received by Dr. Floyd Markham from the United States Bureau of Animal Industry as the fifth chick embryo passage of the original strain.

* The authors wish to thank Dr. Hilary Koprowski for his helpful suggestions, and Donald Hendrickson for his valuable technical assistance.

In this laboratory it was passaged once in chick embryos, then 12 times in duck embryos, then twice in chick embryos. The present designation of MD20Z indicates the total passages of the virus to be 20. Undiluted allantoic fluids make up the virus pool. *Tumor.* The Ehrlich ascites tumor was received through the kindness of Dr. George Klein of the Karolinska Institute, Stockholm, Sweden. The tumor has been continuously passaged in this laboratory since 1950 in Swiss (or ICR) mice every 7 days. In order to test the effect of the virus preparations *in vivo,* female Swiss mice weighing 20-25 g were inoculated i.p. with 0.2 ml of pooled ascitic fluid containing 10-30 million tumor cells. *Virus-tumor cell suspension.* Ascites cells were collected 7-10 days after implantation. washed as previously described(1), resuspended in an arbitrary volume of Parker's synthetic medium 199(4) and the number of cells was counted in a standard bright-line Neubauer hemocytometer. The average of 4 counts was taken in order to determine the cell concentration. In all experiments the final concentration of Ehrlich ascites cells was adjusted with medium 199 to give 5 x 10^6 cells per ml. Penicillin and streptomycin were routinely added to the culture medium to give a final concentration of 100 units and 200 γ per ml respectively. Virus was added as noted in each experiment. Two ml aliquots of the cell-virus suspensions were transferred to 15 x 150 mm pyrex test tubes, which were closed with silicone stoppers, placed in a roller drum and incubated at 37°C for the desired time interval. After incubation the cultures were harvested, pooled and stored in rubber-stoppered vaccine bottles at –25°C until the virus was used in the next tissue culture passage or egg titration was performed. *Egg titrations.* Tenfold dilutions of tissue culture or ascites fluid were made in 2% N-Z† amine solution (Lederle) containing 2 mg of streptomycin per ml. This material was then injected in 0.2 ml amounts into the chorioallantoic cavities of 9-11-day-old embryonated chicken eggs. Eggs were incubated at 37°C, candled daily for seven days, and the number of dead embryos recorded. LD_{50} titers were calculated according to the method of Reed and Muench(5). *Virus identification.* Newcastle immune chicken serum of high titer, prepared in this laboratory, was diluted 1:2 with 2% N-Z amine solution containing 2 mg of streptomycin per ml; mixed with an equal volume of undiluted ascitic or tissue culture fluid and with 10-fold dilutions thereof; and incubated at room temperature for 60 minutes. Eggs were inoculated in groups of 5, incubated at 37°C, and candled as described above. Virus identification was confirmed when protection was afforded by the immune serum mixture in all the dilutions through 10° and no protection was afforded by normal serum in control egg inoculations performed simultaneously. *Mouse inoculation.* At frequent intervals aliquots of 0.5 ml of undiluted tissue culture passage material were inoculated i.p. into a number of Swiss mice which had received transplants of the Ehrlich ascites tumor 4 days earlier. A control group of tumor-bearing mice was given a similar inoculum of uninfected tissue culture material prepared in the same manner and at the same time as the infected cultures. *Analysis of oncolytic effect and viral growth.* Smears of the ascitic fluid from at least 2 experimental and control mice were prepared daily, stained by Leishman's method, and examined. Karyorrhexis of tumor cells or their disappearance from the fluid was regarded as cytological evidence of oncolysis(6). This assessment of oncolysis was confirmed by determination of the survival

† Tryptic digest of casein (Sheffield Chemical Co., N. Y. City) 2% solution in distilled water.

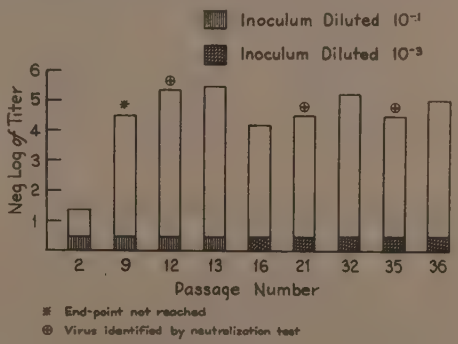

FIG. 1. Egg infectivity titer of NDV-Ehrlich cell tissue culture passages.

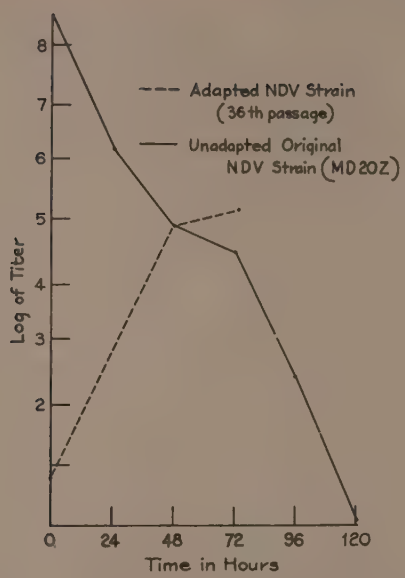

FIG. 2. Virus titer of NDV in Ehrlich cell cultures* after various intervals of incubation.

* Final dilution of MD20Z = 10^{-1} in 5 × 10^6 Ehrlich cells/ml.

ratio of experimental and control groups over a period of 30 days. In certain experiments 3 to 5 mice were killed, the ascitic fluid pooled, and egg infectivity titrations performed. In some instances neutralization tests were also done.

Results. Virus passage. The serial passage of NDV in the Ehrlich ascites cells *in vitro* was begun with a ratio of one egg LD_{50} of the MD20Z strain to 10,000 cells. After 96 hours incubation, the tube contents were harvested and pooled. This pool, in a final dilution of 10^{-1}, served as inoculum for the second passage, which was incubated as before. Starting with the third passage and continuing to the present (38th), incubation has been for 48 hours only. From the 13th passage on, each tissue culture inoculum was a 10^{-3} dilution of the preceding tissue culture passage.

Virus titers at various tissue culture passage levels. Egg infectivity titers were determined at various passage levels. Fig. 1 gives the results of representative titrations, and reveals the uniformity of infectious virus content at levels after the second passage. Thus the 13th and 16th passages contained approximately the same amounts of virus as subsequent passages, although the latter received smaller inocula. Fig. 2 shows the increase, during a period of 48 hours, in egg infectious virus in the 36th tissue culture passage. In contrast, a rapid drop in titer occurred when the unadapted parent NDV strain was inoculated into Ehrlich cell cultures. With the adapted strain there was an increase of approximately 4 logs in the period of 48 hours, whereas the drop in infectivity titer of the unadapted strain over the same period of incubation was 3.5 logs, and no virus was detectable after 120 hours.

Inoculation of ascites-bearing mice with

TABLE I. Summary of Experiments on Ascites-Bearing Mice Inoculated with Newcastle Disease Virus Tissue Culture.

Passage No.	Cytological evidence of oncolysis	Onset of karyorrhexis (days after inoculation of virus)	Survival ratio*	Virus titer after 96 hr
Parent strain	0	—		0
5	0	—	0/20	10^{-4}
10	+	7	0/20	$<10^{-4}$
12	0	—	0/20	$<10^{-4}$
18	+	7	2/20	N.D.
28	++	3	4/20	N.D.
29	+++	3	14/33	[illegible]
37	+++	4	14/22	[illegible]

* No. of mice surviving over No. of tumor-bearing mice.
N.D. = Not done.
All 150 control mice which did not receive virus inoculum died.
+ = Karyorrhexis, but tumor cells persisted until death. ++ = Complete disappearance of tumor cells in 50% of mice. +++ = Complete disappearance of tumor cells in all mice (see footnote ‡).

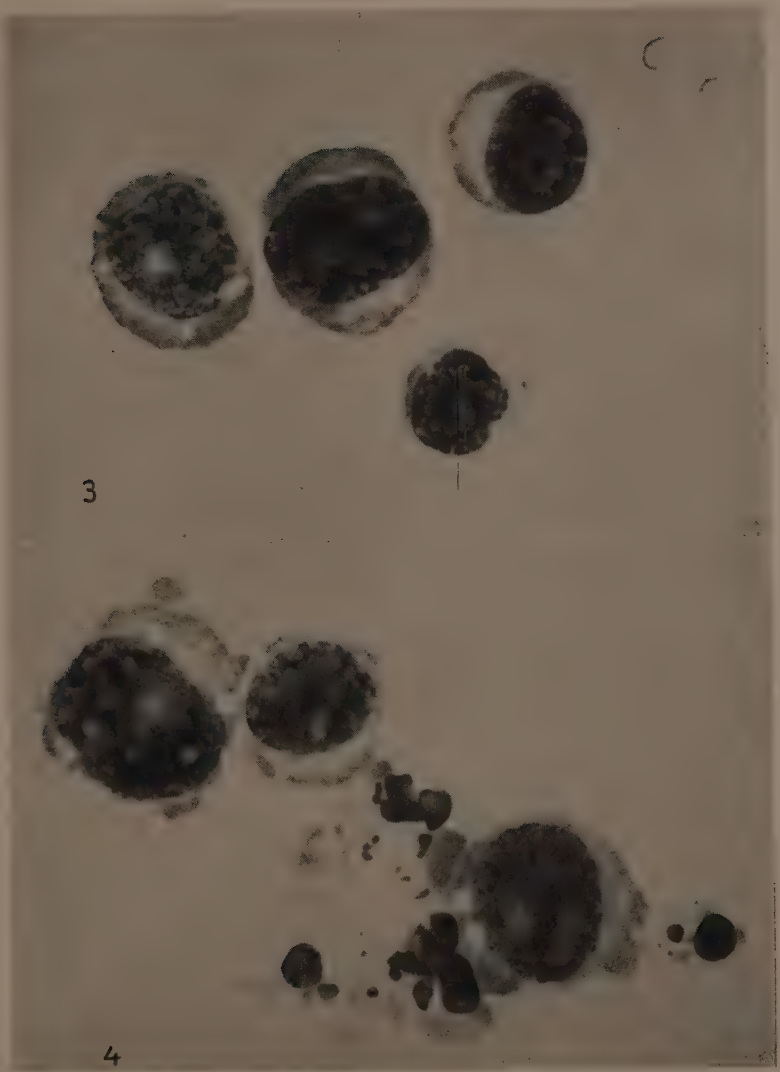

FIG. 3. Ehrlich ascites tumor cells 7 days after inoculation of uninfected tissue culture material. Leishman's stain × 1400.

FIG. 4. Karyorrhexis in 2 Ehrlich ascites tumor cells 7 days after inoculation of 10th passage of NDV in tissue culture. Leishman's stain × 1400.

NDV tissue culture. Virus from 7 different tissue culture passages was injected into mice bearing 4-day ascites tumors. Table I gives a summary of these experiments, which indicated that tissue culture passage of the Ehrlich-adapted strain of NDV enhanced its ability to produce oncolysis. Initially no oncolysis was evident, and it was not until the 10th passage that karyorrhexis was observed. This occurred 7 days after virus inoculation (Fig. 3 and 4). Inocula from later tissue culture passages produced more effective and more rapid oncolysis, and the survival ratios increased (Table I). Karyorrhexis was ob-

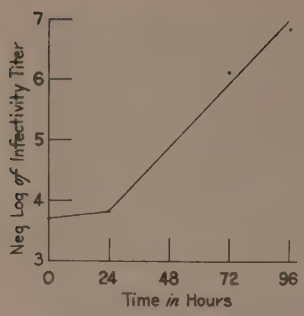

FIG. 5. Increase in egg infectivity titers of ascitic fluid of mice injected with adapted NDV.*

* 29th tissue culture passage.

served 4 days after injection of virus from the 37th tissue culture passage; no tumor cells were seen in the fluid withdrawn from any of the 4 mice on the 7th day after virus inoculation,‡ and the survival ratio was 14/22. No distinction was made between death from ascites and later death from solid tumors at the site of the original tumor injection. No virus could be detected in ascitic fluid obtained 96 hours after injection of the parent NDV strain; after 29 passages of the virus *in vitro* a comparable sample of ascitic fluid titrated to $10^{-6.8}$ in eggs. Fig. 5 shows a 3.5 log increase in egg-infectivity titer of the ascitic fluids obtained daily for 96 hours after injection of virus from the 29th tissue culture passage.

Virus identification. Undiluted tissue culture material from the 12th, 21st and 35th passages, as well as undiluted ascitic fluid obtained from mice 96 hours after injection with the 29th and 36th passages, was completely neutralized by NDV immune serum. Also chorioallantoic fluids withdrawn from embryonated chicken eggs injected with NDV-normal serum controls were spot tested, and gave positive hemagglutination which was specifically inhibited by high titer NDV immune serum.

Discussion. Inhibition of growth of the Ehrlich ascites tumor by the tissue culture adapted strain of NDV is different from the phenomenon described by Moore and Diamond(2). These workers regarded the effect of the virus on tumor cells as comparable to the hemagglutinating action of viruses on red cells(6). They did not observe any cytological evidence of oncolysis like that described above. The cytological changes which follow inoculation of the tissue culture adapted NDV are essentially the same as those which have been described during the destruction of Ehrlich tumor cells after infection with West Nile and Bunyamwera viruses(6,7). Since the latter are not hemagglutinating viruses, the cytolytic action of the adapted strain of NDV is probably of the same nature as that of other oncolytic viruses, and like them depends upon the multiplication of virus in the tumor. This was confirmed by the absence of cytological changes after inoculation of the original strain of NDV, when virus multiplication in the tumor could not be demonstrated; indeed, only the later passages of the adapted virus which multiplied to a high degree were capable of inducing consistent oncolysis.

‡ Less than 1% of tumor cells was observed in one smear, but most of these showed advanced degenerative changes.

Summary. The propagation of NDV in Ehrlich ascites tumor cells maintained *in vitro* is described. After prolonged serial passages *in vitro*, multiplication of the virus occurs in the tumor *in vivo*, and in the later passage levels is accompanied by cytological and biological evidence of oncolysis.

1. Flanagan, A. D., and Colter, J., *Cancer Res.*, in press.

2. Moore, A. E., and Diamond, L. C., *J. Immunol.*, 1953, v71, 441.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 663.

4. Morgan, J. R., Morton, H. J., and Parker, R. C., *ibid.*, 1950, v73, 1.

5. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

6. Love, R., Koprowski, H., and Cox, H. R., *Cancer Res.*, 1953, v13, 350.

7. Orsi, E. V., Love, R., and Koprowski, H., *Proc. Am. Assn. Cancer Res.*, 1955, v2, 38.

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Susceptibility to Poliomyelitis Virus of HeLa Cells Adapted to Growth in Horse Serum Medium. (21946)

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With the development of a subline of HeLa cells capable of growth in medium containing horse serum in place of human serum(1) it was of interest to determine its susceptibility to the effects of poliomyelitis virus.

Materials and methods. HeLa cells adapted to growth in human serum and the subline grown in horse serum were obtained from Dr. Virginia Evans in Dr. Earle's laboratory, National Cancer Institute, in the form of flask cultures on glass. Monkey kidney cultures were prepared according to the trypsinization method of Youngner(2). HeLa cells of both sublines were transferred from large bottles into tubes by mechanical washing of cells off glass with the proper growth media. 0.5 ml of a suspension containing approximately 75,000 cells per ml was inoculated into each tube. The tubes were incubated for 24-48 hours in a stationary position on their sides before use in the tests with virus. The growth medium consisted of 40% Earle's balanced salt solution, 20% ultrafiltrate from chick embryo extract, and 40% serum. At the end of the short growth period these cultures were changed to Eagle's synthetic medium containing 4% horse serum(3), which was quite efficient as a maintenance medium for both HeLa cell lines. Monkey kidney cultures were prepared in roller tubes by growing the original trypsinized kidney suspension in Melnick's "A" medium(4) for 4 days, at which time they were changed to a maintenance medium consisting of 75% Earle's balanced salt solution, 24% ox serum ultrafiltrate, and 1% horse serum. Poliomyelitis viruses used for quantitating the susceptibility of the cell lines were the Mahoney Type I, 6468 Type II (isolated in this laboratory from the stool of a case of paralytic polio), and the Saukett strain of Type III. Titrations of standard virus pools were carried out by inoculating 0.1 ml of each serial 10-fold dilution into each of 3 tissue culture tubes. Dilutions were carried out in Hanks' balanced salt solution. Cultures were read daily by microscopic examination for at least 7 days after inoculation. Stool emulsions prepared by extraction with balanced salt solution containing 2500 units each of penicillin and streptomycin, and clarified by centrifugation at 8000 r.p.m. for 30 minutes in a Spinco centrifuge, were used in a volume of 0.3 ml per tube. The tubes were then placed in the incubator for one hour, after which time the medium containing the stool emulsion was drained and replaced with maintenance medium.

TABLE I. Titrations of Standard Pools of 3 Types of Poliomyelitis Virus in Monkey Kidney Cells and HeLa Cells Grown in Horse Serum and Human Serum Media.

	Poliomyelitis virus type					
	Exp. 1			Exp. 2		
Tissue culture	I	II	III	I	II	III
HeLa						
(Human serum)	5.75*	4.5	5.75	5.75	5.75	5.75
(Horse ")	4.75	3.5	3.75	6.5	5.25	6.25
Monkey kidney	5.75	5.75	5.75	6.5	6.25	6.25

* Expressed as logs of 50% end-points of titrations per 0.1 ml.

Results. In Table I are given the 50% end-point titrations of each of the 3 types of poliomyelitis virus in the 3 different tissue cultures. Results of the first experiment in Table I indicated that the HeLa cells grown in the horse serum medium were less susceptible than were those grown in the human serum or the kidney cells. However, a second experiment indicated an equal susceptibility of all 3 tissue cultures.

In the first of these 2 experiments, on the 5th day following inoculation of the Type I virus at the 10^{-4} dilution when all tubes were showing marked cytopathogenic effect, super-

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TABLE II. Multiplication of Poliomyelitis Virus in HeLa Cells Grown in Human Serum and in Horse Serum.

Tissue culture	Poliomyelitis virus type I	II	III
HeLa (Human serum)	5.75*	6.5	4.75	7.25
" (Horse ")	5.75	6.5	4.75	6.0

* Titration of 50% end-points in logs/0.1 ml in monkey kidney roller tubes.

nates were harvested from both the horse and the human serum HeLa cell lines. These were titrated in monkey kidney roller tubes. In the second experiment similar harvests were made from the 2 HeLa sublines on the 5th day after virus inoculation from the tubes which had received 10^{-5} dilution of Type I, 10^{-3} dilution of Type II, and 10^{-5} dilution of Type III virus. Again, these harvests were titered in monkey kidney roller tubes. The results of these comparisons of the ability of the 2 sublines of HeLa cells to grow poliomyelitis virus are indicated in Table II, and there is no significant difference between the 2, except perhaps in the one experiment where the human serum HeLa cells apparently gave greater multiplication of the Type III virus.

In an attempt to show the relative efficiency of the 2 sublines of HeLa cells in the isolation of polio virus from human materials, a total of 23 stool emulsions previously shown to contain polio virus, were inoculated into the various tissue cultures. These stool suspensions contained an equal number of materials originally positive in either monkey testicle or kidney cultures for each of the 3 types of poliomyelitis virus. In Table III are shown the results which indicate that the HeLa cells grown in horse serum are just as responsive to polio virus in human stools as those grown in human serum.

In Dr. Earle's laboratory this subline of HeLa cells grown in horse serum medium has been adapted to mass growth in a suspended cell shaker flask type of culture(1,5,6). Because of certain advantages for basic studies on interrelationships between virus and cell of such a culture containing large numbers of viable HeLa cells, multiplying while suspended in the culture medium, 2 experiments were carried out to determine polio virus multiplication in these suspended cells. The suspended cells were grown in Dr. Earle's laboratory. Horse serum adapted HeLa cells grown in stationary flasks were used as the inoculum for the shaker flasks. One flask contained over 400,000,000 cells (approximately 1.6 g wet weight) at the time of inoculation, and the second contained 1,000,000.000 cells (approximately 4 g wet weight).

TABLE III. Isolation of Poliomyelitis Viruses from Stools of Patients in HeLa Cells Grown in Human Serum and in Horse Serum.

Tissue culture	Positive stools/total tested Exp. 1	Exp. 2
HeLa (Human serum)	6/10	8/13
" (Horse ")	4/10	10/13
Monkey kidney	—	10/13

The total volume of each culture was centrifuged and the sedimented cells were resuspended in shaker flasks in 150 ml of Eagle's maintenance medium containing 4% horse serum. The first flask was inoculated with 3 ml of the 10^{-1} dilution of our standard Mahoney Type I virus, which has a titer of $10^{7.5}$ per ml. A sample was removed immediately after mixing and again after 24 hours incubation. During incubation the flask was rotated at the rate of 9060 revolutions per hour and was aerated by flushing 4 times with 4 liters of 5% CO_2 in air. The second flask was inoculated with 0.1 ml of the 10^{-3} dilution of the same virus. This flask was rotated at 12,000 r.p.h. and aerated and sampled like the first. Assay of the samples from the first flask carried out in monkey kidney roller tubes showed an increase of virus titer from 10^{5} per ml at zero hour to greater than $10^{7.5}$ in 24 hours. Samples from the second flask titered at 10^{-2} at zero hour and $10^{6.5}$ in 24 hours.

Discussion and Conclusions. Human serum is not readily obtainable in large volumes from a uniform source for use in tissue culture. Furthermore, the possible presence of specific antibodies introduces further difficulties in the use of cells grown in human serum media when working with certain human viruses. The adaptation of HeLa cells to growth in horse serum media eliminates these difficulties.

It would appear from these experiments

that HeLa cells adapted to growth in the presence of horse serum rather than in media containing human serum are quite susceptible to the cytopathogenic effect of all three types of poliomyelitis virus, and are as efficient in producing virus as the HeLa cells grown in the human serum. They likewise seem to be as efficient in their ability to demonstrate the presence of poliomyelitis virus in human stool suspensions. Preliminary evidence indicates that this subline of HeLa cell grown in mass suspended cultures is also capable of supporting the growth of poliomyelitis virus.

1. Perry, V. P., Evans, V. J., and Earle, W. R., *Science*, 1955, v121, 805.

2. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

3. Eagle, H., *ibid.*, 1955, v89, 96.

4. Black, F. L., and Melnick, J. L., *J. Immunol.*, 1955, v74, 236.

5. Earle, W. R., Bryant, J. C., and Schilling, E. L., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1000.

6. Earle, W. R., Bryant, J. C., Schilling, E. L., and Evans, V. J., *Ann. N. Y. Acad. Sci.*, 1955, in press.

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Glycemic Effects of Chlorpromazine in the Mouse, Hamster and Rat. (21947)

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Various pharmacological effects of chlorpromazine (CPZ) have been reported in the literature among which are depression of body temperature, potentiation of morphine and barbiturates, anti-epinephrine as well as anti-acetylcholine action, and a depression of cellular activity or narcobiosis(1,2,3). The drug which structurally is 10-(3-dimethylaminopropyl)-2-chlorophenothiazine hydrochloride is similar in structure to promethazine but shows very little anti-histaminic property. In view of the various profound effects on the nervous system and cellular activity, the question arose as to the possible effects on blood sugar levels during the post-injection period when this substance is pharmacologically most active.

Materials and methods. The effect of intraperitoneal injections of chlorpromazine (Thorazine, S.K.F.) on blood sugar level of non-fasted and fasted white male mice of the Rockland (RAP) strain was first studied. Animals were injected with CPZ at 5 mg/kg body weight and blood samples were withdrawn *via* the orbital sinus(4), at intervals of 30 minutes, and at 1, 2, 3 and 5 hours later. Non-injected controls were bled at the same interval periods and blood sugar determinations similarly made. Since the response to CPZ injection in mice was a substantial wave of hyperglycemia, similar experiments were carried out with the hamster and rat. Also the glycemic effects of chlorpromazine in the alloxan diabetic mouse, and the effects, if any, of this substance on the hypoglycemic action of insulin were observed. Mice were separated into 2 groups, each group consisting of 16 animals. Group I was made diabetic by subjecting it to a 48-hour fasting period followed by injection of alloxan monohydrate, 75 mg/kg *via* the intracardiac route(5). After diabetes had been established, these animals were treated with CPZ and the glycemic effects determined. Group II mice received insulin injections in the following manner: Animals were fasted for 24 hours, injected with CPZ, 5 mg/kg, ip, and 2 hours later were injected subcutaneously with insulin, 1/6 unit per 18 g body weight. A minimum of 16 animals was used in all cases for each type of experiment with mice, hamsters and rats. Blood sugar determinations were made by the Somogyi-Nelson method(6).

Results. Normal male mice receiving CPZ

injections of 5 mg/kg, ip, showed an average progressive increase in blood sugar from 142.2 to 202.3 mg% in 30 minutes which by 3 hours had reached 282.8 mg%. A gradual decline in blood sugar then followed which at 5 hours post-injection time had returned to slightly higher than normal value. Non-treated animals which were bled at the same time intervals as treated ones showed a slight blood sugar elevation at the end of the first hour, but all succeeding blood sugar samples were within the normal range.

The hamster showed a hyperglycemic response to CPZ injections similar to that obtained in the mouse although to a lesser degree. The minimal dose required to produce a significant hyperglycemic response was found to be 10 mg/kg when injected intraperitoneally. Hamsters in both the fasted and non-fasted state showed an elevated blood sugar level following CPZ injection, but the hyperglycemia was more pronounced in those animals which had not been starved. The average 24-hour fasting blood sugar level was found to be 96 mg% and 3 hours after injection of CPZ at 10 mg/kg, ip, the glycemic level was raised to 194 mg%. The non-fasted hamster had a glycemic level of 108 mg% which reached 212 mg% at 3 hours after injection of CPZ.

The rat showed very little if any significant rise in blood sugar following injections of CPZ. Various doses were tried ranging from 5 to 60 mg/kg, the latter being approximately the LD_{50}, and in each case the blood sugar level was not appreciably changed. The average fasting glycemia level in the rat was found to be 89 mg% and the maximal glycemic response produced by any dose level was an elevation to 127 mg%. This occurred at 3½ hours following ip injection of CPZ at 20 mg kg of rat body weight.

Chlorpromazine intensified the fasting hyperglycemia in alloxan diabetic mice and significantly decreased the survival time of these diabetic animals. The average 24-hour fasting blood sugar in the non-treated diabetic mice was 374 mg% while those treated with CPZ showed an elevation to greater than 600 mg% at 2 hours post-injection time. Blood samples were taken in all diabetic mice on alternate days via cardiac puncture with none of the 16 diabetic mice receiving CPZ at 5 mg/kg surviving more than 3 days after such treatment. In contrast to this, 60% of the non-treated alloxan diabetic animals survived a week of alternate day blood withdrawals. In practically all cases of treated and non-treated animals death occurred during the 24-hour starvation period prior to blood sampling. All blood samples were collected in amounts not exceeding 0.2 ml.

Chlorpromazine was observed to afford some protection against the hypoglycemic convulsions and subsequent death produced in mice by injection of 1/6 unit of insulin per 18 g body weight. Of the 16 mice which received insulin at 2 hours after injection of CPZ, 9 animals exhibited convulsions but no deaths occurred in spite of the fact that no glucose was administered. Of the 16 mice receiving the same dose of insulin but no CPZ, 14 convulsed and 11 of these died.

Discussion. It is of interest to speculate on the means by which chlorpromazine brings about so significant a rise in glycemic levels of the mouse and hamster. First, of course, would be the narcobiotic action of this substance which decreases cellular activity so that metabolic oxidation reactions are depressed. This would in effect decrease the amount of glucose being oxidized within the cell and therefore decrease the need or intake of glucose by the cell from extracellular spaces and the blood. As a consequence a rise in blood sugar would occur. Whether or not the depression of cellular activity is the sole means by which CPZ brings about its hyperglycemic effect is difficult to say at this time. The hypothermic effect of this drug, which might be used as an index of cellular activity, is also produced in the rat and yet this animal shows no appreciable hyperglycemic response. The possibility of the endocrine glands such as the adrenals, hypophysis or pancreas acting directly or indirectly through glycogenolysis or gluconeogenesis in the liver has yet to be explored. The profound species variation between the rat and the mouse in the hyperglycemic response to this drug cannot be fully explained at this time. The basal metabolic rate is appreciably higher in the mouse(7),

and this may be the requisite for demonstrating a hyperglycemic response to CPZ since a depression of cellular metabolism in an animal normally functioning at a high metabolic rate would bring about more pronounced changes in the concentration of substances in the blood which are metabolized than would be expected in an animal with a lower normal rate of metabolic activity. Evidence in support of this concept would be the somewhat intermediate effect of CPZ on the blood sugar of the hamster which has a BMR somewhat intermediate to that of the mouse and rat (8,9).

The protection afforded by CPZ from the hypoglycemic convulsions and death following insulin injection in mice would be expected in view of the hyperglycemic state of these animals prior to insulin administration. Whether or not there is any direct antagonism of the insulin molecule cannot be said without further investigation.

Summary. The glycemic effects of chlorpromazine were studied in the mouse, hamster and rat. A hyperglycemic response was found in the mouse and hamster with the mouse having the greater response of the two. No significant glycemic change was observed in the rat. An exacerbation of the diabetic state and decrease in survival time was found in alloxan diabetic mice treated with CPZ. A protection from hypoglycemic convulsions and death produced by insulin was afforded to mice which had been injected with CPZ 2 hours previous to the injection of insulin.

1. Decourt, P., *J. Pharm. and Pharmacol.,* 1954, v6, 491.

2. Kopera, J., Armitage, A. K., *Brit. J. Pharmacol.,* 1954, v9, 392.

3. Holzbauer, M., and Vogt, M., *ibid.,* 1954, v9, 402.

4. Stone, S. H., *Science,* 1954, v119, 100.

5. Lazarow, A., *J. Lab. and Clin. Med.,* 1947, v32, 1258.

6. Somogyi, M., *J. Biol. Chem.,* 1952, v194, 19.

7. Morrison, P. R., *J. Cell. and Comp. Physiol.,* 1948, v31, 281.

8. Herrington, L. P., *Am. Inst. Physics,* 1941, Reinhold Publ. Corp., N. Y.

9. Kayser, C., *Ann. Physiol.,* 1939, v15, 1087.

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Induction of Toxigenicity in Non-Toxigenic Strains of *C. diphtheriae* with Bacteriophages Derived from Non-Toxigenic Strains. (21948)

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Freeman(1,2) reported that some bacteriophages isolated from *C. diphtheriae* could induce totally non-toxigenic strains of *C. diphtheriae* to become toxigenic and that these transformed strains were indistinguishable in all respects from toxigenic strains occurring in nature. This observation has been confirmed in this laboratory(3,4) as well as in other laboratories(5,6). In each instance, toxigenicity was induced only by bacteriophages obtained from naturally-occurring toxigenic strains of *C. diphtheriae.*

Because of the epidemiological significance of this transformation, studies were undertaken to determine the relative frequency with which the change could be produced in non-toxigenic strains isolated from cases and carriers. Of the 52 strains tested so far the change has been induced in only 8 strains, all of which were the mitis or mitis-like type. While Hewett(7) has reported the transformation in gravis-type strains employing phages derived from toxigenic gravis strains. it has not been accomplished in this laboratory. However, definitions of the gravis type differ in different laboratories. During serial passage of one group of cultures with phages from toxigenic strains, complete lysis was obtained with 2 non-toxigenic strains, (A-8888, mitis-like North Dakota, and A-8782, gravis,

Alabama) without any demonstrable change in toxigenicity. It appeared probable that these strains were lysogenic in themselves. Therefore, experiments were planned to determin whether or not the bacteriophages isolated from strains A-8888 and A-8782 might induce toxigenicity in other non-toxigenic strains. Although the data presented here concerning these studies are not complete, sufficient evidence has accumulated to warrant a report.

Materials and methods. Details of the technics used in these experiments will be published elsewhere. The non-toxigenic strains used in these experiments were chosen from among those which had become toxigenic when treated with phages derived from toxigenic strains. All strains of *C. diphtheriae* from which bacteriophage was derived, as well as those strains in which toxigenicity was induced, were tested first for non-toxigenicity by subcutaneous inoculation in guinea pigs, by intracutaneous inoculation of rabbits, and by the *in vitro* toxigenicity plate method. None showed any evidence of original toxigenicity by any of the methods. The bacteriophages were obtained from atoxigenic strains A-8888 and A-8782 (later also A-8188) by inoculating heart-infusion-agar plates with 3 to 4-hour heart-infusion-broth cultures of the organisms, incubating overnight, followed by extraction of the phage by freezing the agar over CO_2 ice and thawing at room temperature. The expressed liquid was centrifuged at high speed and the supernatant fluid, after heating at 52-53° for 20 minutes, was used in serial passage.

Results. Serial passage of non-toxigenic Freeman strains F-324 and F-326, with phages obtained from A-8888 and A-8782, produced almost complete clearing after the third passage. Centrifuged sediments of the lysed broth cultures were streaked on the *in vitro* toxigenicity plate and gave the precipitation lines typical of toxigenic strains of *C. diphtheriae*. A portion of this same sediment, when streaked on heart-infusion-agar plates and incubated overnight, produced partially lysed colonies which also produced lines of precipitation on the *in vitro* toxigenicity plate. Similar centrifuged sediments when injected intradermally into a rabbit, produced reactions which were indistinguishable from those produced by typical, toxigenic strains of *C. diphtheriae*. The same results were obtained with 3 separate phage extracts from strains A-8888 and A-8782.

Attempts to induce toxigenicity in other non-toxigenic strains of *C. diphtheriae* with these same phages were not successful. Curiously, these strains, which failed to become toxigenic when treated with phages from A-8888 and A-8782, did become toxigenic when treated with phages derived from known toxigenic strains. This observation suggests that phages derived from non-toxigenic strains may differ from phages derived from toxigenic strains. However, phages derived from non-toxigenic strains A-8888 and A-8782 and adapted to atoxigenic strains F-324 and F-326 readily induced toxigenicity in non-toxigenic strains A-334, A-8613, and A-8630. The number of passages necessary to bring about this change varied both with the strain and the phage. Other strains are being passed with these phages but the results are incomplete as yet. The toxigenicity of these phage-changed strains has been tested on both the *in vitro* toxigenicity plate and intradermally in the rabbit.

In another experiment phage was isolated from non-toxigenic strain A-8188 by the method previously described. After 44 passages with atoxigenic strains F-324 and F-326 only partial lysis of these strains was observed and toxigenicity could not be demonstrated. The heated supernatants from the last passage were used for treatment of non-toxigenic strains A-8613 and A-8630. Complete lysis was obtained after the seventh passage and the centrifuged sediments were shown to be toxigenic on the *in vitro* plate and when injected intradermally into the rabbit.

The observations made during the course of the above experiments seem to suggest that there may be different races of diphtheria bacteriophage or that several factors are requisite for induction of toxigenicity not all of which are necessarily present in any given strain of *C. diphtheriae* or in any one bacteriophage.

Summary. It has been established that certain bacteriophages derived from naturally-

occurring non-toxigenic strains of *C. diphtheriae* will induce toxigenicity in certain non-toxigenic strains of *C. diphtheriae* as readily as will phages derived from naturally-occurring toxigenic strains.

1. Freeman, V. J., *J. Bact.*, 1951, v61, 675.
2. Freeman, V. J., and Morse, I. U., *ibid.*, 1952, v63, 407.
3. Parsons, E. I., and Frobisher, M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 746.
4. ———, *Am. J. Pub. Health*, 1953, v43, 269.
5. Hewitt, L. F., *Lancet*, 1952, v2, 272.
6. Groman, N. B., *Science*, 1953, v117, 297.
7. Hewitt, L. F., *J. Gen. Microbiology*, 1954, v11, 272.

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Tissue Conversion of Thyroxine to Triiodothyronine.* (21949)

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The isolation of a new thyroxine-like compound from the thyroid and plasma by Gross and LeBlond(1,2) and the subsequent identification of this substance as 3,5,3′-triiodothyronine by Gross and Pitt-Rivers(3,4), and Roche, Lissitsky and Michel(5) have stimulated a great deal of investigative work. Triiodothyronine has been shown to be about 5 times as potent as thyroxine in preventing thiouracil-induced goiter in rats(6,7,8) and to produce a much more rapid elevation in the oxygen consumption of hypothyroid animals and man than does thyroxine(8-11). These findings, along with the earlier reports by Gross and LeBlond(1) of isolation of the I^{131}-triiodothyronine from tissues and plasma of thyroidectomized animals given I^{131}-labeled thyroxine, led Gross and Pitt-Rivers(6) to postulate that thyroxine is enzymatically deiodinated to triiodothyronine in the tissues and that triiodothyronine is the active form of the hormone at the tissue level. Although Albright, Larson and Trust(12) reported the conversion of thyroxine to triiodothyronine by kidney slices and MacLagan and Sprott(13) made similar observations using liver homogenates, Roche, Michel, Michel and Lissitzky (14) have been unable to demonstrate the deiodination of thyroxine to triiodothyronine *in vitro*. More recently Roche and Michel(15), on the basis of *in vivo* work with thyroidectomized animals, found that the thyroid is the sole important source of plasma triiodothyronine and that there was no triiodothyronine or its derivatives in the bile or urine of such animals after the injection of labeled thyroxine. Kalant, Lee, and Sellers(16) reported that triiodothyronine was the major radioactive compound found in the skeletal muscle of propylthiouracil-treated rats given I^{131}-labeled thyroxine but Taurog(17) stated that his group did not find consistent conversion of thyroxine to triiodothyronine in liver, kidney, spleen or diaphragm.

With these conflicting observations in mind, the following experiments were devised in an attempt to demonstrate the *in vivo* conversion of thyroxine to triiodothyronine by the rat.

Methods. (a) Thirty-three male Sprague-Dawley rats, weighing 250 to 300 g and maintained at 80°F and on standard Purina Laboratory Chow were given 10 μc (0.5 to 0.7 μg) of I^{131}-labeled thyroxine† intravenously. (The stock solution of thyroxine consisted of this hormone in 50% propylene glycol in water: aliquots were diluted with 0.9% saline before administration.) At intervals of 30 minutes, one, 3, 6, 12, and 24 hours after injection, liver, kidney, and skeletal muscle were removed. Nine rats were studied at the 30-minute point, 3 at one hour, 7 at 3 hours, 3 at 6 hours, 4 at 12 hours and 7 at 24 hours.

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† Obtained from Abbott Laboratories, Oak Ridge, Tenn.

These tissues were homogenized in NH_4Cl buffer (pH 8.4), boiled for 30 minutes, digested with crude trypsin for 20 hours and extracted 3 times with n-butanol. The butanol extract was then evaporated to dryness and the residue taken up in 2 to 3 ml of 0.5 N NaOH. Identical experiments were performed either using purified trypsin instead of crude trypsin or omitting trypsin digestion entirely with essentially the same results except that digestion with either purified or crude trypsin produced a slightly higher yield of butanol-extractable material. The material taken up in NaOH was then run through kieselguhr chromatographic columns according to the method of Gross and Pitt-Rivers(4)‡ and Braasch, Flock and Albert(18). Butanol containing 20% chloroform equilibrated with 0.5N NaOH was run through the column until thyroxine, triiodothyronine and iodide were eluted. The solvent system was then changed to 10% isopropyl alcohol in butanol for the purpose of recovering di- and monoiodotyrosine. The effluent was collected with a fraction collector and the radioactivity of each fraction was assayed with a scintillation detector. The results were expressed graphically, plotting counts per minute in the fraction versus fraction number. The relative amounts of all radioactive components recovered was determined roughly by comparing the areas under the peaks obtained. (b) An experiment using 16 rats was performed identical to that described above except that 10 μc (0.5 to 0.7 μg) of I^{131}-labeled triiodothyronine was injected into the animals. The animals were sacrificed 1, 3, 6 and 12 hours after the injection of triiodothyronine. Four animals comprised each group. (c) An experiment similar to that described under (a) was carried out in 16 animals 2 weeks after thyroidectomy. In this case 10 μc of thyroxine which had been re-purified by passage of the stock solution through the kieselguhr column was injected intravenously and the animals sacrificed 30 minutes after injection. Specimens of liver, kidney, and muscle were homogenized and

‡ The authors wish to express their gratitude to Dr. Jack Gross for his invaluable help and advice in the use of the kieselguhr chromatographic technic.

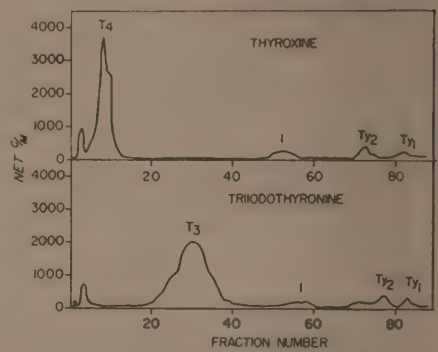


FIG. 1. Representative graphs of kieselguhr chromatographic analysis of liver, kidney and muscle at various time intervals after injection of (a) I^{131}-labeled thyroxine and (b) I^{131}-labeled triiodothyronine. T_4 indicates thyroxine; T_3, triiodothyronine; I, iodide; Ty_1, monoiodotyrosine, and Ty_2, diiodotyrosine.

treated as described above. Other specimens of liver and kidney were homogenized in sucrose solution at 4°C and separated, by means of differential ultracentrifugation(19), into nuclear, mitochondrial, microsomal, and residual protein fractions. The fractions thus obtained were treated as described in the initial part of the experiment and the extracts were subjected to kieselguhr chromatographic analysis.

Recovery studies with either labeled thyroxine or labeled triiodothyronine revealed that 98 to 101% of the radioactivity placed on the column was recovered in the effluent.

Results. Normal rats, whole tissues. Fig. 1 is representative of the results obtained in liver, kidney or muscle from intact normal rats following the administration of labeled thyroxine and triiodothyronine. The chromatographs were essentially alike at all time intervals studied. When thyroxine was administered the main compound recovered corresponded to thyroxine on chromatographic analysis, and in most instances no triiodothyronine was recovered. However, in 20% of the total number of chromatographic analyses, small triiodothyronine peaks were observed following the administration of thyroxine. Fig. 2 characterizes the findings in these instances. The triiodothyronine peak was present at all of the various time intervals following injection, including the 30-minute point

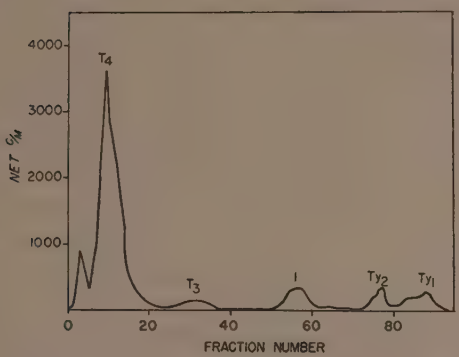


FIG. 2. Plot of radioactivity recovered from kieselguhr columns representative of instances (20% of total assays) where triiodothyronine was recovered from liver, kidney and muscle after injection of I^{131}-labeled thyroxine.

and, although recovered occasionally from all 3 tissues, it was found more frequently in liver and kidney than in muscle. When the approximate relative amount of radioactivity present in each of the chromatographic peaks was determined by measuring the area under each peak, the radioactivity corresponding to triiodothyronine never exceeded 1.5% of the total.

When triiodothyronine was administered, triiodothyronine was the main product of the column separation and no thyroxine was observed at any time. *Thyroidectomized animals*: In the 16 thyroidectomized animals injected with thyroxine and sacrificed 30 minutes later, essentially the same results were observed as in the normal animals. In approximately 20% of all analyses done on liver, kidney and muscle in these animals, small triiodothyronine peaks were detected, the traces of this substance occurring apparently at random in all 3 tissues. In no case did the triiodothyronine peak comprise more than 1.5% of the total radioactivity recovered.

Subcellular fractionations. Fig. 3 is illustrative of the observations made after thyroxine injection into thyroidectomized rats when subcellular fractions (microsomes, mitochondria, nuclei and residual protein) of liver and kidney were subjected to chromatographic separation. Similar findings occurred in the fractions of both liver and kidney. In cases where traces of triiodothyronine were found in the whole homogenates of these tissues, relatively greater amounts of this compound were isolated from some of the subcellular fractions. The greatest concentrations of triiodothyronine appeared in the microsomes and residual fraction of both liver and kidney, the amount of triiodothyronine running as high as 10.5% of the total radioactivity in the case of soluble protein fraction of the kidney. It was of great interest that even in some cases where no triiodothyronine was found in the chromatographic separation of whole homogenates, significant amounts of triiodothyronine were found in the subcellular fractions.

Discussion. By the method of kieselguhr chromatographic separation, we were able to recover triiodothyronine from whole rat tissues following the administration of thyroxine. Triiodothyronine was recovered from liver, kidney and muscle in about 20% of the chromatographic analyses made. When it was found, it was present in only small amounts. never exceeding 1.5% of the total radioactivity recovered. These findings are somewhat at variance with those of Kalant *et al.* with respect to the amounts of triiodothyronine recovered. If thyroxine is actually metabolized to triiodothyronine, and if the appear-

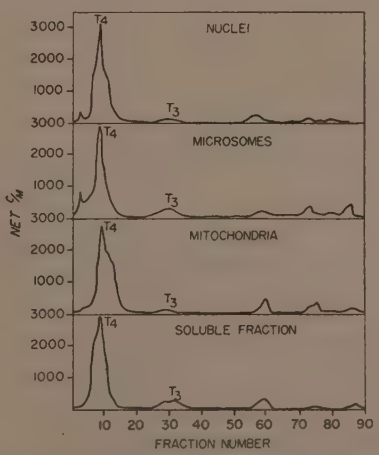
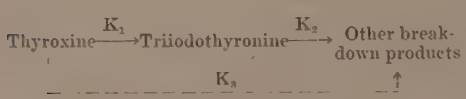


FIG. 3. Radioactivity in effluent from kieselguhr columns representative of findings in subcellular fractions of liver and kidney following injection of I^{131}-labeled thyroxine.

ance of triiodothyronine is not an artifact produced by the conditions of this experiment, it may be that only a relatively small percentage of thyroxine is converted to triiodothyronine. However, since triiodothyronine, once formed, may be further degraded very rapidly, and hence not be recovered from tissue as such, it is not possible to estimate accurately the amount converted.

If such a conversion does actually occur, the mechanism might be simply expressed as follows:



It is probable that rate constant K_2 is at least 3 or 4 times K_1. If this is the case one would expect to find relatively small amounts of triiodothyronine in the tissues involved at any one time, no matter what percentage of thyroxine is degraded via this pathway.

It may be argued that only small amounts of triiodothyronine were recovered and this only relatively infrequently because of poor recovery during the experimental procedure. This point may be answered in part by pointing out that when stock triiodothyronine was subjected to the experimental chromatographic conditions, more than 95% was recovered as triiodothyronine. However, under our experimental conditions we have consistently observed a "front" peak of radioactivity which is recovered from the kieselguhr columns before the thyroxine peak appears. (Figs. 1, 2, 3). Efforts to identify the radioactive components of this peak by paper chromatographic techniques have so far been unrewarding and it is entirely possible that small amounts of triiodothyronine may be included in this material. This might explain the appearance of triiodothyronine, following the administration of thyroxine, at some times and not at others.

Similarly, it may be argued that the appearance of triiodothyronine after administration of thyroxine was due to deiodination produced by the conditions of the experiment rather than to tissue activity. However, when thyroxine stock solution was subjected to the experimental conditions no triiodothyronine was recovered.

The appearance of triiodothyronine in the subcellular fractions in greater concentrations than in the whole homogenate is as yet unexplained. The largest amounts appeared in the microsomes and residual fraction in both liver and kidney, probably due to concentration in these fractions. Concentration alone, however, does not explain the consistent appearance of triiodothyronine in the subcellular fractions in contrast to its irregular appearance in the whole homogenate, since the concentration in all cell fractions was higher than that observed in the whole homogenate. It was noted during the course of our experiments that the "front" peak was consistently smaller in the instances where the subcellular fractions were subjected to kieselguhr chromatographic separation than was the case with the whole homogenate, probably due to minor alterations in the experimental conditions. If this "front" contains appreciable amounts of triiodothyronine this difference might explain the more constant appearance of this substance in the analyses where the "front" was smaller.

Our observations have indicated that a small amount of thyroxine may be converted to triiodothyronine in rat tissues. However, it seems less likely that the main mode of action of thyroxine involves this step. In view of the recent works of Thibault and Pitt-Rivers(20), the possibility seems stronger that both substances exert their action only after further alteration, perhaps involving conversion to the acetic acid or other desamino analogues.

Summary. (1) I^{131}-labeled thyroxine was administered intravenously to normal and thyroidectomized rats and the animals sacrificed at varying time intervals thereafter. Specimens of liver, kidney and muscle were homogenized, extracted with butanol and the extracts were subjected to kieselguhr chromatographic analysis. Small amounts of triiodothyronine were recovered from all 3 tissues in about 20% of the analyses carried out. (2) When triiodothyronine was administered and the tissues subjected to the same conditions, no thyroxine was recovered. (3) When

the tissues of rats given thyroxine were homogenized and subjected to fractionation into their subcellular components, triiodothyronine was more readily recovered than was the case where the whole homogenate was used. A possible explanation for this is discussed.

1. Gross, J., and LeBlond, C. P., *Endocrinology*, 1951, v48, 714.
2. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 688.
3. Gross, J., and Pitt-Rivers, R., *Lancet*, 1952, v262, 439.
4. ———, *Biochem. J.*, 1953, v53, 645.
5. Roche, J., Lissitzky, S., and Michel, R., *Compt. rend. Acad. Sc.*, 1952, v234, 1228.
6. Gross, J., and Pitt-Rivers, R., *Lancet*, 1952, v262, 593.
7. ———, *Biochem. J.*, 1953, v53, 652.
8. Tomish, E. B., and Woollett, E. A., *Lancet*, 1953, v264, 726.
9. MacLagan, N. F., Sprott, W. E., and Wilkinson, J. H., *ibid.*, 1952, v263, 915.
10. Heming, A. E., and Holtkamp, D. E., *Fed. Proc.*, 1953, v12, 330.
11. Gross, J., Pitt-Rivers, R., and Trotter, W. R., *Lancet*, 1952, v262, 1044.
12. Albright, E. C., Larson, F. C., and Trust, R. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 137.
13. MacLagan, N. F., and Sprott, W. E., *Lancet*, 1954, v267, 368.
14. Roche, J., Michel, R., Michel, D., and Lissitzky, S., *Compt. rend. Soc. Biol.*, 1951, v145, 228.
15. Roche, J., and Michel, R., *Acta Endocrinologica*, 1954, v17, 385.
16. Kalant, H., Lee, R. B., and Sellers, E. A., *Endocrinology*, 1955, v56, 127.
17. Taurog, A., *The Thyroid*, Brookhaven Symposia in Biology, 1954, No. 7, 88.
18. Braasch, J. W., Flock, E. V., and Albert, A., *Endocrinology*, 1954, v55, 768.
19. Lee, N. D., and Williams, R. H., *ibid.*, 1954, v54, 5
20. Thibault, O., and Pitt-Rivers, R., *Lancet*, 1955, v268, 285.

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Intracellular Potassium Concentration in Normal and Diabetic Rat Liver.* (21950)

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It has previously been shown that marked abnormalities of carbohydrate metabolism occur in liver slices from normal animals incubated under conditions leading to lowered intracellular potassium concentration(1). Thus, potassium depletion leads to decreased glycogen deposition from glucose and to decreased overall glucose utilization, while gluconeogenesis from pyruvate and other precursors is markedly increased. These metabolic abnormalities are qualitatively similar to those found in liver slices from diabetic rats even in the presence of normal intracellular potassium concentration(2,3). Furthermore, a number of observations have been made, both clinically and in experimental animals, suggesting that potassium depletion may lead to disturbances of carbohydrate metabolism qualitatively similar to those of diabetes mellitus(4,5). It seemed of interest, therefore, to determine whether lowered intracellular potassium concentration occurred in diabetic liver *in vivo*. Accordingly, electrolyte, water and glycogen analyses were made on diabetic and normal rat livers.

Material and methods. Male rats weighing 250 g were made diabetic by the intravenous injection of alloxan (40 mg per kilo). Three to 4 weeks later these animals, as well as normal controls, were fasted for 8 hours (water *ad lib.*), then sacrificed for blood and liver analysis. The animals were sacrificed under nembutal anesthesia by severing the carotid

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‡ R. Samuel McLaughlin Traveling Fellow in Medicine.

TABLE I. Blood Analysis on Normal and Diabetic Rats.

Group	Blood sugar, mg/100 ml blood Mean	Range	Na (mEq/L serum)	K (mEq/L serum)
Normal (11)	99	78-117	149.1 ± 6.4*	4.74 ± 1.5*
Diabetic (11)	292	121-470	144.7 ± 3.8	4.25 ± 1.8

* Mean values ± stand. error of mean.

arteries. The livers were removed, blotted free of blood, and 2 aliquots were taken. One was for glycogen analysis(6) and the other was used to determine water, sodium and potassium. This second aliquot was dried, defatted and extracted with 0.75 N HNO_3 as described by Lowry and Hastings(7). Sodium and potassium were determined by flame photometry. Whole blood was also analyzed for glucose(8) and blood serum for sodium and potassium.

Results. Table I shows the blood sugar and serum sodium and potassium values for 11 normal and 11 diabetic animals. The serum sodium and potassium concentrations were slightly lower in the diabetic than in the normal group. These data were used in calculating the intracellular potassium concentrations.

In Table II are recorded the analytical data obtained on the normal and diabetic livers, together with calculated values for extracellular water $(H_2O)_e$, intracellular water $(H_2O)_c$ and intracellular potassium concentration $[K]_c$. From the analytical data it may be seen that no statistically significant difference was observed in the concentrations of water, potassium or sodium between the normal and diabetic livers.

Using these data, and the assumption that the sodium of liver is entirely extracellular, the extracellular water $(H_2O)_e$ and intracellular water $(H_2O)_c$, in g per kilo of fat free tissue, were calculated according to methods previously described(7). No difference in either $(H_2O)_e$ or $(H_2O)_c$ was found between the livers of the normal and diabetic groups.

After making allowance for the amount of potassium in the extracellular water of the tissues, the concentration of potassium in the intracellular water $[K]_c$ was calculated. These values proved to be 187 mEq per kilo of intracellular water for the normal livers and 184 mEq per kilo of water for the diabetic livers. The difference is without significance.

Discussion. Since abnormalities in carbohydrate metabolism similar to those encountered in diabetic liver have been observed in normal liver where intracellular potassium concentration is experimentally reduced below normal, we have felt it important to determine whether the intracellular potassium concentration of liver is or is not reduced below normal in diabetes. From the data presented above it would appear that the intracellular potassium concentration is quite normal in diabetic liver. It would seem, therefore, that the metabolic abnormalities which have been described in hepatic tissue for diabetic animals are not attributable to a reduction in potassium concentration but rather it appears more likely that potassium depletion and insulin de-

TABLE II. Analytical Data on Normal and Diabetic Rat Livers. Mean values are expressed per kg of fat-free tissue (F.F.T.)—standard error of the mean.

Group	H_2O, g	K, mEq	Na, mEq	Glycogen, g
Normal (11)	715 ± 1.5	96.2 ± .6	31.4 ± 2.1	7.0 ± 1.2
Diabetic (11)	723 ± 2.8	95.5 ± 1.0	30.5 ± 1.2	19.4 ± 2.2

Derived data

	$(H_2O)_e$,* g/kg F.F.T.	$(H_2O)_c$,† g/kg F.F.T.	$[K]_c$, mEq/kg $(H_2O)_c$	$[K]_c$, mEq/100 g F.F. solids
Normal (11)	198 ± 15	517 ± 15	187 ± 8	33.8 ± .2
Diabetic (11)	198 ± 7	515 ± 7	184 ± 4	33.3 ± .2

* $(H_2O)_e$—g of extracellular H_2O per kg of F.F.T., calculated on the assumption that liver Na is extracellular.

† $(H_2O)_c$—g of intracellular H_2O per kg of F.F.T., calculated from total tissue H_2O and $(H_2O)_e$.

ficiency affect intracellular metabolism similarly but independently.

Summary. The intracellular potassium concentration was found to be normal in diabetic rat liver.

1. Hastings, A. B., Teng, C. T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, 1952, v194, 69.

2. Renold, A. E., Teng, C. T., Nesbett, F. B., and Hastings, A. B., *ibid.*, 1953, v204, 533.

3. Hastings, A. B., Renold, A. E., and Teng, C. T., *Trans. Assn. Am. Phys.*, 1953, v66, 129.

4. Gardner, L. I., Talbot, N. B., Cook, C. D., Bermenand, H., and Uribe, R., *J. Lab. and Clin. Med.*, 1950, v35, 592.

5. Eliel, L. P., Pearson, O. H., and White, F. C., *J. Clin. Invest.*, 1952, v31, 419.

6. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, v100, 485.

7. Lowry, O. H., and Hastings, A. B., *ibid.*, 1942, v143, 257.

8. Somogyi, M., *ibid.*, 1945, v160, 61.

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Renin and Antirenin in Treatment of Long Term Experimental Renal Hypertension in the Dog.* (21951)

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A number of reports have suggested that the pathogenesis of experimental renal hypertension changes with time. Thus it has been suggested that in acute experimental renal hypertension the pathogenesis is renal and in chronic experimental renal hypertension the pathogenesis is extrarenal or (according to one group of investigators) neurogenic. There have been no reported studies on the pathogenesis of long term experimental renal hypertension (*i.e.*, hypertension of 4 or more years duration) in the dog. Thus Ogden and colleagues(1,2) showed that certain sympatholytic and general anesthetic drugs decreased the blood pressure of chronic renal hypertensive rats but not of acute renal hypertensive rats. They also showed that the removal of a unilaterally constricted kidney in the rat caused a decrease in blood pressure in acute but not in chronic renal hypertensive rats. On the basis of these findings, Ogden suggested that the pathogenesis of experimental renal hypertension changes with time from a renal pressor mechanism to a neurogenic mechanism. Likewise, several groups of British investigators have reported that complete nephrectomy in the acute renal hypertensive rat produces a decrease in blood pressure, whereas there is no decrease during survival in the chronic hypertensive rat(3,4). These results are similar to those of earlier workers who reported that total nephrectomy in renal hypertension of one week's duration in the rabbit results in normotension but after 2 to 4 months of hypertension, total nephrectomy results in persistence of the hypertension during survival(5,6). Floyer and others have interpreted these findings as demonstrating an extrarenal mechanism for the pathogenesis of chronic experimental renal hypertension. Moss and Wakerlin(7) and Page(8) however, found no significant difference in the effects of sympatholytic and general anesthetic drugs in acute and chronic experimental renal hypertension in dogs. Moss and Wakerlin considered acute experimental renal hypertension in dogs to last 6 months following renal artery constriction and most of their acute hypertensive dogs were 3 to 6 months hypertensive. Since then, Haynes and Dexter(9) showed that an increase in plasma renin following renal artery constriction in the dog persists for some weeks and Shorr and colleagues(10) showed that increased plasma VEM is balanced by VDM 8 to 12 weeks following renal

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artery constriction in dogs. These observations suggest that the dividing line between acute and chronic experimental renal hypertension in dogs may be 3 rather than 6 months. In keeping with this, Ohler and Wakerlin(11) found that dibenzyline produced no decrease in the blood pressure of 7 renal hypertensive dogs within 3 months following renal artery constriction whereas a decrease occurred in dogs which were hypertensive for 6 months or more. However, the decrease was not to the normotensive level and certainly not to the hypotensive level produced by dibenzyline in normotensive dogs. Recently, Ogden and colleagues(12) reported that pentobarbital decreased the blood pressure of dogs with renal hypertensions of more than one year's duration but not to normotension. These findings suggest that vasomotor tonus is in abeyance during acute renal hypertension but returns to at least a normal level during chronic renal hypertension in the dog.

To study the pathogenesis of long term experimental renal hypertension in dogs, we observed the effect of semipurified hog renin therapy and of passive immunization with antirenin to hog renin in dogs with renal hypertensions of 50 to 121 months duration. Two years ago, our research group showed that a course of semipurified hog renin from renal cortex protected against the development of experimental renal hypertension in dogs and was effective in the treatment of chronic experimental renal hypertension of 8 to 37 months duration in dogs, whereas numerous control tissue extracts were without effect(13). Since the effectiveness of prophylaxis and treatment was well-correlated with the serum antirenin titre, the findings were considered good evidence for the hypothesis that renin (or a closely related renal protein) plays the principal pathogenetic role in experimental renal hypertension in the dog for at least the first 3 years. A pathogenetic role for renin is also suggested by the antihypertensive effect of homologous antirenin to hog renin passively transferred to renal hypertensive dogs(14).

Methods. Ten dogs with renal hypertensions of 50 to 121 months duration were treated for 6 to 12 months with one or two courses of daily intramuscular injections of semipurified hog renin from cortex in doses varying from 2.5 to 20.0 D. U. (Goldblatt) per kg. The semipurified hog renin was prepared by a modification of the method of Marshall and Wakerlin(15) or the method of Haas and Goldblatt(16). The purity of the renin varied from 16-175 D. U. per mg N, but most of it was 75-175 D. U. per mg N. Antirenin titres on the serum were determined monthly by a method already described(13). The time between courses of hog renin therapy in the 6 dogs receiving a second course of hog renin was at least 6 months, during which the blood pressure reached its pretreatment hypertensive level and the antirenin titre fell to 0. Male and female dogs were made hypertensive by a standardized modification of the Goldblatt technique of renal artery constriction already reported(13). Mean blood pressure readings were obtained by femoral artery puncture one or 2 times per week, with occasional comparison by the strain gauge technic. Studies of blood urea nitrogen, urinalyses, and determinations of body weight and clinical condition were made at monthly intervals or more often when indicated.

For the passive transfer study, 3 of the dogs with renal hypertensions of 93 to 108 months duration were given high titre homologous antiserum containing antirenin to hog renin. Each of these dogs had previously been treated with a course of semipurified hog renin with a sufficient lapse of time (6 months or more) to permit a decrease in the antirenin titre to 0 and a return of the blood pressure to the pretreatment hypertensive level for 2 to 3 months. Blood pressure was measured every other day for one week prior to antirenin administration and the antirenin titre was determined to be 0 A. U. per cc of serum. Antirenin in total amounts varying from 947 A. U. to 3,560 A. U. per kg was administered subcutaneously in 4 divided doses over a 2-day period. Blood pressures and serum antirenin titres were determined on the 3rd, 5th, and 7th days following the first day of antirenin administration and subsequently at weekly or semi-weekly intervals. Urinalyses were made prior and subsequent to the administration of antirenin and the clinical condition of the dogs

TABLE I. Antihypertensive Effect of Semipurified Hog Renin from Cortex in Experimental Renal Hypertension in 10 Dogs with Hypertensions of 50 to 121 Months Duration.

Dog No.	Sex	Max anti-renin titre (A.U./cc)	Duration of hyper-tension	Blood pressure in mm Hg: Normotension	Hypertension	Max re-sponse to treatment	Effect on hypertension, %
1	♀	37	50	125 ± 7.0*	183 ± 3.7*	151 ± 5.8*	−55
2	♀	29	51	122 ± 7.8	162 ± 5.4	153 ± 7.2	−23
3	♂	32	51	123 ± 5.0	167 ± 7.5	129 ± 8.0	−86
4	♂	0	51	115 ± 5.4	201 ± 6.9	219 ±10.5	+21†
5	♀	50	56	127 ± 6.8	158 ± 3.8	142 ± 4.8	−50
6	♂	24	60	120 ± 6.8	185 ± 4.0	140 ±10.5	−69
7	♀	18	66	117 ± 5.2	185 ±10.5	138 ± 8.4	−68
8	♂	20	67	127 ± 9.1	172 ± 5.5	147 ± 6.6	−55
2	♀	68	69	122 ± 7.8	172 ± 4.8	148 ± 4.0	−48
9	♂	22	71	117 ± 4.6	164 ± 9.4	145 ± 7.6	−47
7	♀	21	81	117 ± 5.2	166 ± 6.7	140 ± 3.9	−53
9	♂	85	82	117 ± 4.6	170 ±11.0	142 ± 6.6	−53
8	♂	16	87	127 ± 9.1	166 ± 5.8	150 ± 6.6	−41
3	♂	40	98	123 ± 5.0	171 ± 7.0	147 ± 9.1	−50
10	♂	0	99	129 ± 9.0	160 ± 2.7	183 ± 7.2	+74†
10	♂	11	121	129 ± 9.0	166 ± 3.2	146 ± 5.9	−54

* Avg ± stand. dev.
† Maintained chronic pressor effect in the absence of antirenin.
The P value (t test) was <.01 in all experiments.

was observed daily. Control experiments with normal serum were conducted.

Results. Table I summarizes results with hog renin injections. The normotensive and hypertensive blood pressure levels are each based upon a minimum of 12 to 18 readings over a period of 2 to 3 months . The maximal response to treatment level is based on a minimum of 6 to 12 readings over a period of one to 2 months. All of the dogs showed a significant antihypertensive effect ranging from 23 to 86% reduction in their hypertensions, except the 2 animals which did not develop antirenin. None of the dogs showed adverse effects from treatment. Appetite, body weight, and general clinical condition were well maintained. Urinalyses and blood urea nitrogen determinations consistently gave normal findings.

The maximal antihypertensive effect for the first course of injections occurred during the third to the sixth months of treatment as the antirenin titre approached its maximum and was maintained until treatment was discontinued. The maximal antihypertensive effect for the second course was observed during the second to the fourth months of treatment, correlating with the more rapid anamnestic development of antirenin. Following the end of treatment, the blood pressure gradually returned to its pretreatment level over a period of 2 to 6 months as the antirenin titre fell toward 0. Fig. 1 shows a typical antihypertensive effect from a course of semipurified hog renin in a long term renal hypertensive dog. The 2 dogs which did not develop anti-

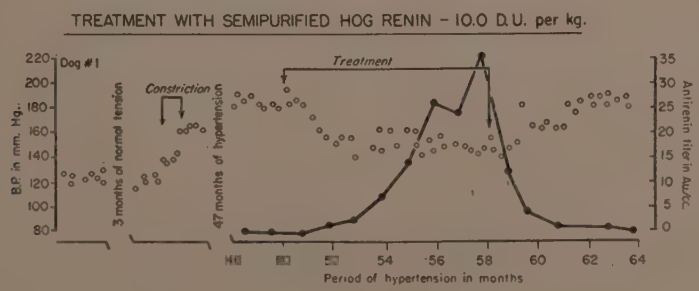

FIG. 1.

TABLE II. Antihypertensive Effect of Homologous Antirenin to Hog Renin Passively Transferred to Dogs with Long Term Experimental Renal Hypertension.

Dog No. (see Table I)	Sex	Total antirenin dose (A.U./kg)	Max antirenin titre	Duration of hypertension	Blood pressure in mm Hg: Normotension	Blood pressure in mm Hg: Hypertension	Blood pressure in mm Hg: Max response to antirenin	Effect on hypertension, %
7	♀	3560	9	93	117 ± 5.2*	169 ± 11.6*	128	− 79
7	♀	947	5	97	117 ± 5.2	177 ± 9.0	102	−100
7	♀	0	0	99	117 ± 5.2	166 ± 8.7	164 ± 7.0	− 4*
9	♂	1353	4	96	117 ± 4.6	164 ± 4.0	133	− 66
10	♂	1700	15	108	129 ± 9.0	164 ± 2.5	114	−100
10	♂	0	0	114	129 ± 9.0	166 ± 5.2	166 ± 3.0	0*

* Normal serum controls.

renin not only did not show an antihypertensive effect but showed a maintained chronic pressor effect which has been discussed elsewhere(17).

Table II summarizes results with the passive transfer of antirenin. The 4 passive transfer experiments with antirenin produced 66 to 100% reductions in the hypertensions of the three long term renal hypertensive dogs compared with antihypertensive effects in the same dogs of 54 to 68% as a result of 5 courses of hog renin. The maximal antihypertensive effect was obtained on the third or fifth day of the passive transfer experiment, usually simultaneously with the maximum antirenin titre. The blood pressure then gradually returned to the previous hypertensive level over a period of two weeks or more as the antirenin titre fell toward 0. Control passive transfer experiments with normal serum in 2 of the 3 dogs were without antihypertensive effect. None of the animals showed adverse changes in clinical condition or abnormalities in urinalyses following antiserum or serum administrations. Fig. 2 illustrates a passive transfer of antirenin and a control experiment in one of the dogs.

Discussion. The results with hog renin and with the passive transfer of antirenin support the hypothesis that renin (or a closely related renal protein) plays a significant role in experimental renal hypertension of many years duration in the dog. Although our research group has already shown that numerous control tissue extracts are without antihypertensive effect(13), the lack of response in the 2 long term hypertensive dogs failing to develop antirenin on hog renin therapy, further rules out a non-specific foreign protein effect as the cause of the antihypertensive response. The failure of most of the long term hypertensive dogs to show a decrease in blood pressure to normotension whereas most of the dogs with chronic hypertensions of 8 to 37 months showed such a decrease on semipurified hog renin therapy(13), suggests at least three possible explanations: (1) the pathogenesis of experimental renal hypertension in the dog may undergo a gradual change with the passage of years whereby renin plays a decreased role; (2) progressive arteriolosclerosis may develop over a period of years; and (3) compensatory mechanisms opposing the antihypertensive effect of actively produced antirenin may be enhanced in long term as contrasted with chronic experimental renal hypertension in the dog. The decrease in blood pressure to normotension or near normotension produced by the passive transfer of antirenin is opposed to (1) and (2). Likewise an extensive study of the passive transfer of antirenin in renal hypertensive dogs to be published later(18) indicates that the duration of chronic hypertension is without effect on the antihypertensive response to passive immunization with antirenin. Moreover, histological examination of the arterioles of long term hy-

PASSIVE TRANSFER OF ANTIRENIN

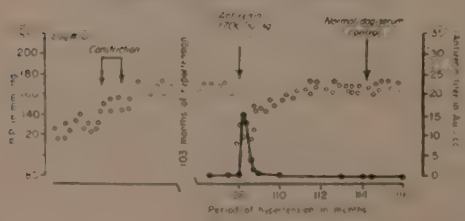

FIG. 2.

pertensive dogs by us confirmed the previous observation of Goldblatt that experimental renal hypertensive dogs show a minimal amount of arteriolosclerosis. This leaves the suggestion that compensatory hypertensive mechanisms come more prominently into play when serum antirenin is gradually increased by semipurified hog renin than when the serum antirenin titre is quickly developed by passive immunization with antirenin. Our observations shed no light on what these compensatory mechanisms may be although increased vasomotor tonus may be involved. Recently our research group reported an increase in renal renin concentration proportional to the antirenin titre in normotensive and renal hypertensive dogs on hog renin therapy but found no difference in this respect among acute, chronic, and long term renal hypertensive dogs(19).

Floyer(20) has suggested that possibly renin may exercise its pathogenetic role in chronic experimental renal hypertension by acting on the kidney perhaps to decrease the production of the blood pressure regulatory or depressor renal hormone (loss of which by complete nephrectomy results in renoprival hypertension)(21). Conceivably this action might evolve gradually during acute experimental renal hypertension and become significant only in chronic experimental renal hypertension. This hypothesis would reconcile the conflicting findings reviewed in the introduction of this report and would be in agreement with our finding that renin still plays a dominant role in the pathogenesis of long term experimental renal hypertension. The decreased antihypertensive response of long term experimental renal hypertension to hog renin injections might be due to failure of the blood pressure regulatory renal hormone to increase to the normal level as the serum antirenin titre is gradually increased by active immunization with hog renin. Certainly this intriguing hypothesis should be investigated.

Summary and conclusions. 1. Courses of intramuscular injections of semipurified hog renin from cortex administered to dogs with experimental renal hypertensions of 4 or more years duration produced a significant reduction in blood pressure in those dogs which developed antirenin but not to normotension as previously found in dogs with chronic renal hypertension of 6 to 37 months duration. The 2 dogs which did not develop antirenin showed a maintained chronic pressor effect. 2. Passive immunization with homologous antirenin to hog renin produced a reduction in blood pressure to normotension or near normotension in dogs with experimental renal hypertensions of more than 7 years duration. 3. The mechanism of the decreased response to hog renin therapy in long term experimental renal hypertension in the dog is unknown. The results with passive immunization suggest that a lessened pathogenetic role for renin is not involved nor is progressive arteriolosclerosis. Possibly compensatory hypertensive mechanisms are more effective in the face of the gradually increasing antirenin titre of hog renin therapy than of the abruptly produced antirenin titre of passive immunization. 4. The results indicate that renin (or a closely related renal protein) is the principal pathogenetic factor even in experimental renal hypertension of many years duration in the dog.

1. Reed, R. K., Sapirstein, L. A., Southard, F. D., and Ogden, E., *Am. J. Physiol.*, 1944, v41, 707.

2. Ogden, E., Collings, W. D., and Sapirstein, L. A, *Spec. Pub. N. Y. Acad. Sc.*, 1946, v3, 153.

3. Floyer, M. A., *Clin. Sc.*, 1951, v10, 405.

4. ———, Ciba Foundation Symposium on Hypertension, Boston, 1954, p. 155, Little, Brown & Co.

5. Pickering, G. W., *Clin. Sc.*, 1945, v5, 239.

6. Daniel, P. M., Prichard, M. M. L., and Ward-McQuaid, J. N., *ibid.*, 1954, v13, 247.

7. Moss, W. G., and Wakerlin, G. E., *Am. J. Physiol.*, 1950, v161, 435.

8. Page, I. H., *Factors Regulating Blood Pressure*, Trans. First Conf., New York, Josiah Macy, Jr. Found., 1947, p18.

9. Haynes, F. W., and Dexter, L., *Am. J. Physiol*, 1947, v150, 190.

10. Shorr, E., *Hypertension*, A Symposium, Univ. of Minn. Press, 1950, p86.

11. Ohler, E. A., and Wakerlin, G. E., *Factors Regulating Blood Pressure*, Trans. 5th Conf., New York, Josiah Macy, Jr., Found., 1951, p111.

12. Mandel, M. J., Greene, R. W., Sapirstein, L. A., and Ogden, E., *Am. J. Physiol.*, 1954, v176, 352.

13. Wakerlin, G. E., Bird, R. B., Brennan, V. B., Frank, M. H., Kremen, S., Kuperman, I., and

Skom, J. H., *J. Lab. and Clin. Med.*, 1953, v41, 708.
14. Kuperman, I., and Wakerlin, G. E., *Fed. Proc.*, 1953, v12, 80.
15. Marshall, J., and Wakerlin, G. E., *ibid.*, 1949, v8, 106.
16. Haas, E., Lamfrom, H., and Goldblatt, H., *Arch. Biochem. and Biophysics*, 1953, v42, 368.
17. Wakerlin, G. E., Kremen, S., Frank, M. H., Schmid, H. E., and Graham, L., *Circulation Res.*, 1954, v2, 201.
18. Wakerlin, G. E., Kuperman, I., Schmid, H. E., and Graham, L., to be published.
19. Schmid, H. E., and Wakerlin, G. E., *Fed. Proc.*, 1955, v14, 132.
20. Floyer, M. A., personal communication, 1955.
21. Grollman, A., Muirhead, E. E., and Vanatta, J., *Am. J. Physiol.*, 1949, v157, 21.

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Technic for Preparation of Glycerol-extracted Whole Frog Hearts for Study of Myocardial Fiber Responses.* (21952)

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Since the demonstration by Szent-Györgyi that glycerinated skeletal muscle fibers contract on exposure to adenosine triphosphate, interest has developed in this preparation as a model for the study of muscle contraction (1). Attention has been directed largely to glycerinated psoas muscle fibers because of their length and relative absence of connective tissue(2-4). Glycerol-treated cardiac muscle fibers are more difficult to handle and have not been extensively studied. However, Taeschler and Bing(5) were able to prepare and observe glycerol-extracted trabecular fibers of dog hearts. The purpose of this communication is to describe a glycerinated frog-heart preparation which is suitable for the study of contraction-relaxation cycles.

Method. The frog heart has no coronary circulation, so that tissue nutrition depends upon the diffusion of solutes through the endocardium. The quantity of diffusion is enhanced by numerous fissures in the ventricular walls(6). Therefore, thorough glycerine extraction of the whole heart should be achieved by perfusion and immersion in a glycerol solution. After extraction, ventricular perfusion with suitable solutes should produce contraction of the muscle fibers. Large bullfrogs (*Rana catesbiana*) are employed. For the preparation of glycerinated hearts, the central nervous system is destroyed and the thorax opened ventrally. The pericardium is excised and the heart is dissected from the mediastinum, care being taken to avoid injury to the ventricle. The fresh heart is placed in Ringer's fluid at room temperature and allowed to beat for several minutes until largely cleared of blood. The heart is then tied to an applicator stick by a ligature tightly drawn around the aortae and upper portion of the atria. A small wire hook is inserted through the ventricular muscle near the apex; the free end of the hook is tied to the stick so as to apply a slight stretch to the ventricle. A needle introduced through the atrium permits the infusion of 50% glycerol into the heart until it is moderately distended. This procedure removes all blood, and the tissue becomes translucent. The mounted specimen is then placed in a capped test tube containing 50% glycerol and is stored at −30 to −10°C for 48 to 72 hours. A thin coat of ice may form at the glycerol-air interface; the tissue should be kept below this level.

Results. After extraction for 2 to 3 days, contraction and relaxation of the glycerinated hearts may be produced by using the perfu-

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† National Heart Institute Fellow in Cardiology, 1954-55.

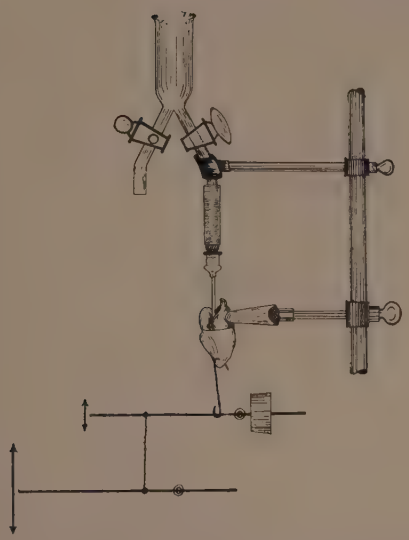

FIG. 1. Diagrammatic sketch of perfusion apparatus with glycerinated frog heart in place. Rate of perfusion of "saline" may be regulated from 30 ml funnel by the stopcock. The counterweighted compound lever system has a net wt of about 800 mg; the system is readily moved by the ventricular muscle fibers.

sion procedure. For this purpose a simple apparatus is employed (Fig. 1). The stick is cut above and below the atrial ligature and the tie holding the hook is severed. The heart is suspended in the apparatus by a pinch clamp applied to the atrium adjacent to the AV groove. The lower end of the hook engages a compound lever system the movements of which are plotted on BMR paper mounted on a kymograph. Writing levers or smoked drums may be used if desired. The heart is perfused by means of a 15-gauge needle inserted through the atrium into the ventricular cavity; the fluid escapes from the ventricle through the small orifice made by the hook. Thorough perfusion of the entire ventricle must be obtained in order to induce maximum contractions. We have found that adequate distention and uniform perfusion of the specimen is assured when the needle is placed through the atrium medial to the bulbus cordis, and a few millimeters into the ventricular chamber. Moderate distention of the tissue probably enhances diffusion of solutes around muscle fibers, and facilitates contraction by stretch of the fibrils(5). The glycerinated myocardium is friable and caution must be observed to prevent tearing of the tissue.

A solution of 0.15 M KCl and 0.02 M $MgCl_2$ (or other molarities as desired) in glass-distilled water serves as the basic perfusate. This solution is similar to that described by Bozler(4), and is designated as "saline." The suspended heart is irrigated with the "saline" at room temperature for several minutes before the administration of ATP. The di-sodium salt of adenosine triphosphate (Sigma) prepared in "saline" is administered through the perfusion funnel. Concentrations ranging between 0.05% and 4.0% ATP have been used. However, reproducible maximal contractions more often occur with 0.5 to 1.0% ATP solutions. Because of the acidity of ATP, it is our custom to adjust the ATP-saline solution to physiological pH range. The pH adjustment may be carried out with N/10 KOH immediately before use.

More than 130 glycerinated frog heart preparations have been studied using this technic. Marked contraction develops within 1 minute from the beginning of ATP perfusion and peak tension occurs within 3 minutes in most instances. We have repeatedly noted

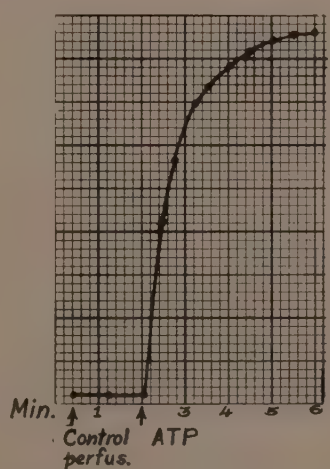

FIG. 2. Example of contraction-curve of a glycerinated whole frog heart. Basic perfusate of KCl .003 M and $MgCl_2$ .0004 M. 1% ATP in basic perfusate added at indicated point.

that ATP contractions of the preparations are enhanced by permitting the specimens to come nearly to room temperature before they are detached from the applicator sticks and set up for perfusion.

Comment. This model system may be of value in *qualitative* studies of contraction and relaxation phenomena in glycerinated cardiac muscle. Observations concerning mechanisms of relaxation are in progress. The rapidity of the diffusion of solutes around the muscle cells is confirmed by the rapidity of ATP response. The ease of operation of the model system and the simplicity of preparation commend this technic for experimental observations of myocardial contractile proteins.

Summary. A method is described for the study of contraction-relaxation phenomena in glycerol-extracted cardiac muscle. Excised hearts of bullfrogs are mounted and extracted in 50% glycerine; subsequently, perfusion with electrolyte solutions and ATP may be carried out, and the contraction recorded by a mechanical system. The ease of preparation and the dependability of the muscle model may prove to be of value in the study of glycerol-extracted heart muscle.

1. Szent-Györgyi, A., *Biol. Bull.*, 1949, v96, 140.
2. Korey, S., *Biochim. and Biophys. Acta*, 1950, v4, 58.
3. Ranney, R. E., *Am. J. Physiol.*, 1954, v178, 99.
4. Bozler, E., *ibid.*, 1951, v167, 276.
5. Taeschler, M., and Bing, R. J., *Circulation Res.*, 1953, v1, 129.
6. Holmes, Samuel J., *Biology of the Frog*, 4th Ed. New York, Macmillan Co., 1927.

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Effect of Lipemia and Heparin on Free Fatty Acid Concentration of Serum in Humans.* (21953)

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The discovery that the *in vitro* clearing of lipemia by plasma obtained after injection of heparin involves lipolysis of triglyceride with release of free fatty acid(1-5) has directed attention to the possibility that free fatty acid might be released *in vivo* by the action of post-heparin clearing factor. Furthermore, the possibility that clearing factor may participate in normal fat metabolism and transport, even when heparin has not been injected (6-8), suggests that free fatty acid may play a physiological role.

It was shown(9) that in rats the free fatty acid concentration of the serum rose when heparin was injected or when alimentary lipemia was present. The present study extends these observations to human subjects in whom lipemia was induced both by the alimentary route and by intravenous injection of fat emulsion and in whom heparin was injected intravenously both in the fasting state and during alimentary lipemia.

Subjects, materials and methods. Ten healthy young adult males, conscientious objectors assigned to the Metabolic Research Division of this laboratory, served as subjects. Heparin (Testagar, 10 mg/ml) was injected intravenously at a dose of 0.5 mg/kg body weight. For the production of alimentary lipemia commercial cottonseed oil (Wesson) was used. The fat emulsion used for intravenous injection (Lipomul, Upjohn) had the following composition: cottonseed oil 15%, dextrose 4.2%, soybean phosphatide 1%, and polyethoxy-propylene oxide (Pluronic F-68, Wyandotte) 0.5%. *Blood lipid analyses.* Venous blood samples were taken from the antecubital vein in chilled syringes and placed immediately in an ice water bath.

* The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

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TABLE I. Blood Lipid Levels of Human Subjects, Fasting, Lipemic, and after Injection of Heparin.

	Fasting	Lipemic	Lipemic, post-heparin	Fasting, post-heparin
	Optical density			
Whole serum				
Mean	.072	.211	.158	
Stand. dev.	.018	.086	.091	
Infranatant				
Mean	.044	.055	.046	
Stand. dev.	.006	.009	.009	
	Total fatty acid (meq/l)			
Whole serum				
Mean	14.0	17.7	15.8	
Stand. dev.	4.68	6.81	3.35	
Infranatant				
Mean	12.1	13.1	14.4	
Stand. dev.	1.69	2.30	2.73	
	Free fatty acid (meq/l)			
Whole serum				
Mean	.51	.75	1.76	1.00
Stand. dev.	.11	.14	.51	.18
Infranatant				
Mean	.51	.75	2.49	
Stand. dev.	.11	.14	.87	

Refrigeration was maintained during centrifugation and separation of the serum up to the time of analysis. Optical density was determined at 700 mμ in a Coleman Junior spectrophotometer using microcuvettes. Total fatty acid was determined by a modification of the method of Smith and Kik(10), and free fatty acid was determined by a modification(9) of the method of Davis(11). In the study of alimentary lipemia analyses were done on whole serum and on the infranatant obtained by high speed centrifugation. The high speed centrifugation was carried out in a Servall superspeed centrifuge in a room maintained at 5°C. After 30 minutes centrifugation at 14,000 rpm the infranatant was drawn off with a syringe and needle and recentrifuged in a similar fashion.

Results. Alimentary lipemia and heparin injection. After a 12-hour overnight fast the 10 subjects received 150 ml of cottonseed oil by fine polyethylene nasogastric tube. Four hours later heparin was given intravenously in a dose of 0.5 mg per kg of body weight. Blood samples were drawn immediately before giving the oil (fasting sample), 4 hours later, just before giving the heparin (lipemic sample), and 15 minutes after the heparin injection (lipemic post-heparin sample). The results are summarized in Table I. The lipemic samples showed an elevation of the values for optical density and free and total fatty acid when compared with the fasting samples. The elevation of free fatty acid concentration was statistically highly significant (Table II). Comparing the values for whole serum and infranatant, it is seen that whereas total fatty acid concentration was significantly reduced by removal of visible turbid lipid, free fatty acid concentration remained unchanged (Table II). Injection of heparin resulted in a decrease in optical density and total fatty acid concentration but a marked elevation of free fatty acid concentration (Table I). The values for free fatty acid concentration of the infranatant samples after heparin injection were even higher than those of the corresponding whole serum. This is attributable to the delay in analysis (about 2 hours) that was required to accomplish the centrifugation. Whole serum post-heparin samples held at 5°C but not centrifuged showed a similar rise in this period of time but pre-heparin samples did not. Thus, in contrast to our previous findings on rat serum(9), refrigeration does not prevent completely *in vitro* lipolysis in human post-heparin serum. For this reason the values for free fatty acid in post-heparin serum given in this paper cannot be taken to represent the true *in vivo* situation since they include an increment that has occurred *in vitro*.

Heparin injection in fasting subjects. Blood samples were taken 15 minutes after intravenous injection of 0.5 mg heparin per kg body weight while the 10 subjects were in the fasting state. Free fatty acid concentration (Table I) was substantially elevated (1.00 meq/l) as compared with the pre-injection control (0.51 meq/l). Because of the possibility that *in vitro* lipolysis was occurring in these post-heparin samples, it cannot be determined how closely this reflects the true *in vivo* situation.

Intravenous injection of fat emulsion. Nine of the test subjects each received 3 separate 500 ml infusions of fat emulsion at 3 different rates on consecutive days. The rates were 30, 60 and 120 drops per minute. The assignment of the order of infusion rates to the subjects

TABLE II. Significance of Differences in Table I.

Difference between	Mean difference	Stand. error of mean difference	t	P
Total fatty acid				
Lipemic, whole serum vs. infranatant	4.58	1.52	3.01	<.01
Free fatty acid				
Fasting, whole serum vs. infranatant	.004	.008	.50	>.05
Lipemic, *Idem*	.008	.007	1.14	>.05
Whole serum, fasting vs. lipemic	.244	.043	5.67	<.01

followed a Latin square design. Venous blood samples were taken at the end of the infusion.

The results are summarized in Fig. 1. Optical density and free and total fatty acid concentrations bore a linear relation to the logarithm of the infusion rate. Analysis of variance showed that the free fatty acid concentrations were significantly different at the various infusion rates and that the regression of free fatty acid concentration on logarithm of infusion rate did not deviate significantly from linearity. In a separate study it was found that injection of the emulsifying agents without fat did not produce any alteration in optical density or free and total fatty acid concentration(18).

Discussion. The results of the present study confirm the findings of the previous study in rats(9) in showing that an increase in free fatty acid concentration of the serum occurs during lipemia. In addition it was found that the free fatty acid resides in the non-particulate portion of the serum since its concentration was not reduced by removal of the turbid lipid particles by high speed centrifugation. Serum albumin is known to have a high binding capacity for fatty acid(12-16) and it is probable that most of it is so bound in serum. It has been demonstrated in this Laboratory that when C^{14}-labeled triglyceride is incubated with post-heparin plasma *in vitro* incorporation of activity into the albumin fraction, as detected by filter paper electrophoresis, occurs(17). The highest concentration of free fatty acid encountered in this study (excluding the heparin studies where *in vitro* lipolysis occurred) was 1.46 meq/l in one of the subjects after rapid intravenous injection of fat emulsion. Assuming a molar concentration of albumin in serum of 0.7 mM/l. this would represent a 2 to 1 molar ratio of fatty acid to albumin. *In vitro* studies (12) have shown that the binding capacity of albumin for fatty acid may go as high as 6 to 1. Although the present study was complicated by the occurrence of *in vitro* lipolysis in the post-heparin samples, it is reasonable to assume that the entire elevation was not due to this factor and that heparin injection in human subjects produces an elevation of *in vivo* free fatty acid concentration comparable to that which has previously been demonstrated in rats(9). Much higher concentrations of total fatty acid were achieved in the studies with intravenous infusion of fat emulsion than in those on alimentary lipemia. It was found that the concentration of free fatty acid rose as the concentration of total fatty acid rose. This suggests that the rate of

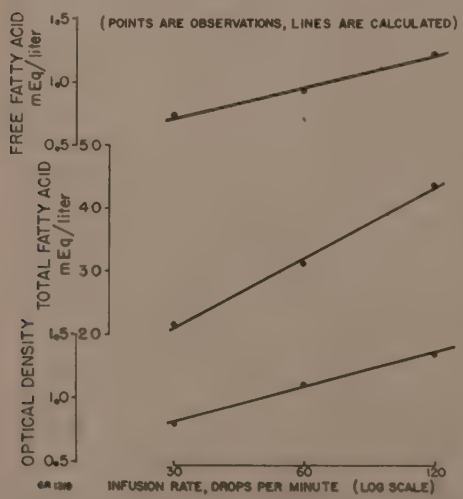

FIG. 1. Relation between rate of infusion of fat emulsion and blood lipid levels at end of infusion in human subjects.

lipolysis is augmented by mass action as the concentration of substrate (triglyceride fat) increases. The findings of this study are consonant with the hypothesis that lipolysis plays a physiological role in removal of turbid fat particles from the blood and that unesterified fatty acid bound to protein may be a mechanism for transporting fat from blood to tissues. Heparin probably accelerates the activity of the lipolytic mechanism(5).

Summary. 1. During alimentary lipemia and lipemia produced by intravenous injection of fat emulsions there was a rise in the concentration of free fatty acid in serum. The free fatty acid was found in the non-turbid portion of serum (infranatant of high speed centrifugation). Injection of heparin produced an elevation of free fatty acid concentration which was greater in lipemic than in fasting subjects. A portion of this elevation after heparin was attributable to *in vitro* lipolysis. 2. It is suggested that lipolysis with free fatty acid formation may play a role in normal fat metabolism and transport.

1. Nichols, A. V., Freeman, N. K., Shore, B., and Rubin, L., *Circulation,* 1952, v6, 457.

2. Shore, B., Nichols, A. V., and Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

3. Nichols, A. V., Rubin, L., and Lindgren, F. T., *ibid.,* 1954, v85, 352.

4. Grossman, M. I., Stadler, J., Cushing, A., and Palm, L., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 132.

5. Korn, E. D., *Science,* 1954, v120, 399.

6. Bragdon, J. H., and Havel, R. J., *Am. J. Physiol.,* 1954, v177, 128.

7. Levy, S. W., and Swank, R. L., *J. Physiol.,* 1954, v123, 301.

8. Pierce, F. T., *Metabolism,* 1954, v3, 142.

9. Grossman, M. I., Palm, L., Becker, G. H., and Moeller, H. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 312.

10. Smith, M. E., and Kik, M. C., *J. Biol. Chem.,* 1933, v103, 391.

11. Davis, B. D., *Arch. Biochem.,* 1947, v15, 351.

12. Gordon, R. S., Jr., Boyle, E., Brown, R. K., Cherkes, A., and Anfinson, C. B., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 168.

13. Klotz, I. M., and Ayers, J., *Trans. Faraday Soc.,* 1953, v13, 189.

14. Robinson, D. S., and French, J. E., *Quart. J. Exp. Physiol.,* 1953, v38, 233.

15. Teresi, J. D., and Luck, J. M., *J. Biol. Chem.,* 1952, v194, 823.

16. Davis, B., and Dubos, R., *Arch. Biochem.,* 1946, v11, 201.

17. McDaniel, R. A., and Grossman, M. I., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 442.

18. Moeller, H. C., Haynes, R. C., Bernstein, L. M., Levy, L. M., and Grossman, M. I., *Med. Nutrition Lab. Rep.* No. 163, 1955.

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Effect of Triiodothyronine on Oxygen Consumption of Tissues Not Responsive to Thyroxine.* (21954)

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The most prominent effect of thyroxine injection in experimental animals is an increase in metabolic rate. Corresponding increases in oxygen consumption have been obtained with such tissues as heart, liver, kidney, diaphragm, skeletal muscle and salivary gland removed from thyroxine-injected animals. In contrast, many other tissues do not participate in the metabolic stimulation, notably brain, spleen, testis, prostate. seminal vesicle, ovary, uterus, thymus and lymph node(1,2).

Since the discovery of triiodothyronine (TRIT) as a form of thyroid hormone even more potent than thyroxine(3), it has been proposed that the latter may be active only after partial deiodination to triiodothyronine (4). Evidence is accumulating that such a transformation does occur(5,6), although its obligatory character is far from established (7). It seemed possible that the lack of response of the tissues mentioned above, as far as thyroxine was concerned, might be due to

* This work was supported by a grant from the Smith, Kline & French Foundation.

TABLE I. Effect of Injections of L-Thyroxine or of L-Triiodothyronine on Tissue Metabolism of Thyroidectomized Rats.

Tissue	Thyroidect.,* control Qo_2	% change from control Qo_2		
		Thyroxine,* 2 mg/kg/d	TRIT,* .5 mg/kg/d	TRIT,† 2 mg/kg/d
Liver	1.02	+ 42.2	+ 44.1	+ 63.8
Diaphragm	.62	+ 43.2	+ 32.3	+ 47.7
Salivary gland	1.98	+ 24.7	+ 37.9	+ 49.1
Heart	.58	+132.4	+135.9	+200.3
Brain	1.34	− .7	− 1.5	+ 2.4
Spleen	1.42	− 4.9	+ 5.6	+ 1.6
Testis	.62	+ 12.9	+ 8.1	− 5.7
Seminal vesicle	.89	+ 1.1	− 4.5	− 3.8
Prostate	1.45	+ 6.2	− 6.2	+ 4.0
Ovary	1.49	+ 4.7	+ 3.4	+ 1.7
Uterus	1.12	− 11.6	− 14.3	+ 7.7
Thymus	1.14	+ 5.3	+ 3.5	+ 4.8
Lymph node	1.17	− .9	+ 11.1	+ 2.5
Gastric Sm. Mus.	.73	+ 2.7	.0	− 3.9

* Five ♂ and 5 ♀ in each of these groups.
† One ♂ and one ♀ in this group.

inability to carry out this transformation. The results of the present work show that, although TRIT is some 4 times as effective as thyroxine in increasing metabolism of several tissues, its results are not qualitatively different from those of thyroxine.

Methods. Both male and female albino rats of the Sprague-Dawley strain were used. All were thyroidectomized at least 4 weeks prior to being injected. As a reference material, L-thyroxine† was injected in a dose of 2 mg/kg/day for 4 days and the animals sacrificed on the fifth day. Preliminary studies indicated that an equimolar dose of L-triiodothyronine‡ produced a much greater elevation in metabolism of heart, liver, diaphragm and salivary gland than resulted from thyroxine. Since one-quarter of the equivalent dose gave approximately the same effect as thyroxine, this amount was used. The compounds to be injected were dissolved in a small amount of 0.1 N NaOH, which was diluted to a final concentration of 0.02 N and was made isotonic with NaCl. Some of the control animals were injected with an equivalent amount of the alkaline saline; since no differences were observed from the uninjected, all control animals were lumped together. Preparation of each of the various tissues was carried out as discussed previously(1,2). Oxygen consumption of each tissue was measured in a Krebs' Ringer phosphate medium with glucose (100 mg%) as substrate.

Results. Table I shows the average oxygen consumptions for the array of tissues studied from the control thyroidectomized animals and from those injected with thyroxine and triiodothyronine. Five males and 5 females were used in each category of control and thyroxine-injected or TRIT-injected. The averages of all tissues not sex-specific are therefore from 10 animals. It can be seen that, amongst the tissues used as references, liver and heart were increased about the same amount by both substances at the dose levels employed. Triiodothyronine accelerated salivary gland metabolism somewhat more, and diaphragm less than did thyroxine. Two additional rats are included which received 4 times as much triiodothyronine as the others, or a dose equimolar with the thyroxine. The oxygen consumption increases were greater in these 2, but not proportionally.

The remaining data in the Table deal with tissues previously found unresponsive to thyroxine, with one exception. These were again unaltered by thyroxine and in addition were not changed by either the 0.5 or the 2.0 mg dose of triiodothyronine. The one exception

† L-Thyroxine was obtained from the Travenol Laboratories, through the kindness of Dr. H. A. Fevold.

‡ L-Triiodothyronine was furnished by Dr. A. E. Heming of Smith, Kline & French Laboratories.

was gastric smooth muscle which had previously been reported as stimulated by thyroxine injection(1). When neither TRIT nor thyroxine influenced its metabolism in the present series, the original data of Barker and Klitgaard were carefully re-examined, but no explanation could be discovered for the discrepancy. Preliminary experiments with several other thyroxine analogs have yielded results similar to those shown here. This tissue now becomes consistent with other unresponsive structures also made up primarily of smooth muscle, such as uterus and seminal vesicle.

One may conclude from these data that, in the thyroidectomized rat, triiodothyronine produces qualitatively the same effects on tissue metabolism as thyroxine. As already mentioned, there is no clear evidence that formation of the triiodinated form from the tetraiodinated is an obligatory step in the tissue response to the latter. The present study discloses that, even if triiodothyronine is made available, some tissues do not respond. An interesting point to pursue would be to determine whether any of these resistant tissues is actually capable of deiodinating thyroxine.

Discussion. Unpublished observations of Klitgaard and Barker indicate that after injection of these amounts of thyroxine, the hormone is present in all tissues to about the same concentration, except for liver and kidney, where levels are much higher. Comparable data are not available for triiodothyronine. At much lower dose levels, both forms of the hormone have been shown to be present in many tissues of the animal body(8). The question of penetration of cells by thyroxine or TRIT has not been answered clearly although Gross *et al.*(8,9), have demonstrated considerable concentration of TRIT by parts of the brain, especially posterior pituitary.

Summary. 1. L-Triiodothyronine (TRIT) injected into thyroidectomized rats at a dose level equivalent to 0.5 mg L-thyroxine/kg body weight/day is capable of stimulating the oxygen consumption of liver, diaphragm, heart and salivary gland preparations to the same extent as 2 mg of L-thyroxine. 2. Neither 0.5 nor 2 mg TRIT injected/kg/day altered the metabolic rates of tissues found unaffected by 2 mg of L-thyroxine, including brain, spleen, testis, seminal vesicle, prostate, ovary, uterus, thymus, lymph node and gastric smooth muscle. 3. Even if thyroxine is converted to TRIT before exerting its effect on the metabolic rate the failure of these tissues to respond to thyroxine does not seem referable to any possible inability to carry out this deiodination.

1. Barker, S. B., and Klitgaard, H. M., *Am. J. Physiol.*, 1952, v170, 81.

2. Barker, S. B., and Schwartz, H. S., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 500.

3. Gross, J., and Pitt-Rivers, R., *Lancet*, 1952, vI, 439, 593; Roche, J., Lissitzky, S., and Michel, R., *Compt. rend. Acad. Sci.*, 1952, v234, 997.

4. Gross, J., and Pitt-Rivers, R., *Biochem. J.*, 1953, v53, 652.

5. Flock, E. V., and Bollman, J. L., *Fed. Proc.*, 1954, v13, 209.

6. Albright, E. C., Larson, F. C., and Tust, R. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 137.

7. Hogness, J. R., Van Arsdel, P., Jr., and Williams, R. H., *J. Clin. Endocrinol. and Metab.*, 1954, v14, 772.

8. Gross, J., in *The Thyroid, Brookhaven Symposia in Biology*, 1955, No. 7, 102.

9. Gross, J., Ford, D. H., and Posner, M., *J. Clin. Endocrinol. and Metab.*, 1955, v15, 836.

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Comparison Between Changes in Serum Inorganic Phosphorus Induced by Glucose and Glucagon in Diabetics. (21955)

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Kirtley, Waife and Peck(1) first reported the effect of glucagon upon blood sugar and serum inorganic phosphate in diabetics. In 4 "stable" diabetics, blood sugar rose to approximately the same level as in normal subjects, remained elevated longer and the serum inorganic phosphorus fall was impaired. In 4 "unstable" diabetics, there was a lesser blood sugar increase and the return to basal levels was about the same as in normals; phosphorus fall was the same as in normals in this group. Kirtley *et al.*(2) extended these observations and showed with epinephrine results similar to those obtained with glucagon.

In the following investigation, we wished to inquire whether diabetics, classified according to the Delta G/Delta P test (based on magnitude of serum inorganic phosphorus fall after intravenous injection of glucose) presented differences in regard to response to glucagon.

Material and methods. The tests were carried out in 35 diabetic patients chosen at random, most of them without previous insulin treatment. A few of the subjects, who were being treated with long acting insulin preparations, were given regular insulin instead for the 2 days preceding the tests. The patients were kept fasting from the night preceding the test and remained at rest for a half hour before and during the test. The Delta G/Delta P test was performed according to a previously described technic, consisting essentially in the intravenous injection of 1 ml/kg body weight of a 50% glucose solution at the rate of approximately 10 ml/m; samples of blood are taken at 0, 30, 45 and 60 minutes. Blood sugar was determined by the Nelson-Somogyi method(3) and serum inorganic phosphorus by the Fiske-Subbarow method(4), modified for the spectrophotometer. According to previous investigations(5,6), an increase in blood sugar at 45 minutes over the initial level (Delta G) of more than 50 mg/100 ml is evidence of a decrease in the subject's capacity to remove glucose from the blood stream, and a decrease in serum inorganic phosphorus below the initial level at 45 minutes (Delta P) of less than 0.30 mg/100 ml is considered defective. A normal Delta P is presumptive evidence of insulin secretion.

TABLE I. Changes in Serum Inorganic Phosphorus (mg/100 ml) after Administration of Glucose in Diabetics. Limiting value between the two groups has been taken as −0.30 mg/100 ml decrease from the initial value at 45 min.

Averages and standard errors.

Group	No. of cases	Initial values	30′	45′	60′
Defective △ P	19	3.14 ± .13	−.07 ± .04	−.09 ± .01	−.06 ± .02
Normal △ P	16	3.42 ± .22	−.31 ± .01	−.50 ± .04	−.45 ± .06

TABLE II. Changes in Blood Sugar after Administration of Glucose (mg/100 ml) in Diabetics Classified According to Serum Inorganic Phosphorus Fall in the Same Test.

Averages and standard errors.

Group	No. of cases	Initial values	30′	45′	60′
Defective △ P	19	236 ± 18	+143 ± 7	+112 ± 8	+87 ± 7
Normal △ P	16	133 ± 12	+131 ± 9	+ 96 ± 9	+75 ± 9
"t" values		4.8*	1.1	1.3	1.1

* Statistically significant difference.

*The author is grateful for the technical assistance of Mr. Andrés Gerardi and Mrs. Esperanza de Manz.

TABLE III. Changes in Serum Inorganic Phosphorus (mg/100 ml) after Glucagon in Diabetics Classified According to Phosphorus Fall after Glucose.
Averages and standard errors.

Group	No. of cases	Initial values	15′	30′	45′	60′
Defective △ P	19	3.08 ± .14	−.47 ± .07	−.53 ± .07	−.40 ± .05	−.30 ± .04
Normal △ P	16	3.37 ± .13	−.57 ± .02	−.72 ± .02	−.54 ± .09	−.39 ± .14
"t" values		1.5	1.3	2.8*	1.4	.7

* Statistically significant difference.

TABLE IV. Changes in Blood Sugar (mg/100 ml) after Glucagon in Diabetics Classified According to Their Phosphorus Fall after Glucose.
Averages and standard errors.

Group	No. of cases	Initial values	15′	30′	45′	60′
Defective △ P	19	232 ± 18	+32 ± 5	+46 ± 3	+48 ± 5	+42 ± 6
Normal △ P	16	137 ± 14	+35 ± 2	+54 ± 3	+53 ± 4	+42 ± 5
"t" values		4.1*	.5	1.8	.7	0

* Statistically significant difference.

The patients were classified according to their Delta P and were then administered glucagon solution in a fashion similar to the administration of glucose; 0.01 ml of glucagon solution per kg body weight was given intravenously, without dilution, and an additional 15 minute sample of blood was obtained. According to specifications given by the manufacturer, the glucagon solution which was used (lot 208-158B-214) contained 0.95 mg of protein/ml, with 0.25% phenol, pH 3. Purity, as compared to crystalline glucagon was 50%; 0.2 μg per kg injected intravenously to an anesthetised cat caused a maximum increase in blood sugar of 30-40 mg per 100 ml 10 to 15 minutes after the injection. The content of insulin was between 0.005 and 0.05 U/ml of solution. The solution was generously furnished by the Eli Lilly Co.

Results. In Table I are shown the changes in serum inorganic phosphorus in the group of diabetics studied, divided according to the fall following glucose.

In Table II may be seen the changes in blood sugar after glucose in both groups.

There is no statistically significant difference with respect to blood sugar rise following glucose between the two groups. The initial level was higher in the group with defective Delta P.

Table III demonstrates serum inorganic phosphorus response to glucagon in the groups classified according to their Delta P after glucose. At 30 minutes, there is a statistically significant difference between the 2 groups,

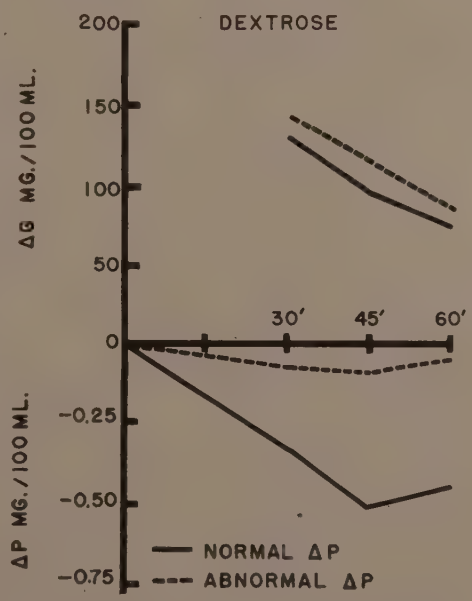

FIG. 1. Changes in blood sugar (△ G) and in serum inorganic phosphorus (△ P) after intravenous administration of dextrose in diabetics classified according to their phosphorus fall in the same test (limiting value between the 2 groups = −0.30 mg/100 ml).

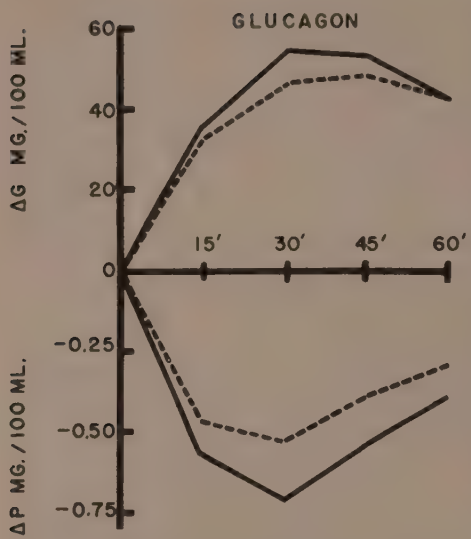

FIG. 2. Changes in blood sugar and serum inorganic phosphorus after intravenous inj. of a glucagon solution in diabetics classified according to their serum inorganic phosphorus response to dextrose. The groups are the same as those indicated in Fig. 1.

the group with "normal" phosphate fall after glucose showing a greater phosphate fall after glucagon. In Table IV the changes of blood sugar after glucagon in both groups are shown. The results are summarized in Fig. 1 and 2.

In Fig. 3 is shown the correlation between maximal Delta P produced by glucagon and maximal Delta P determined by glucose. No definite correlation between the two sets of values appears to exist.

Discussion. The serum inorganic phosphorus fall after glucagon is in general agreement with that following glucose; the fall after glucagon is lesser in the abnormal Delta P group, particularly in the 30-minute sample, where the difference is statistically significant.

The fact that glucagon is able to produce a serum inorganic phosphorus fall in the group where such a fall does not occur after glucose is interesting. It is not likely that this effect is due to insulin contamination, since each cc of the solution contains only between 0.05 and 0.005 U/ml and the amount injected is 0.01 ml per kg. The phosphorus falls found with glucagon are comparable or greater than those found in diabetics after 0.1 U of insulin per kg(7). It would then seem that glucagon has an action of its own upon

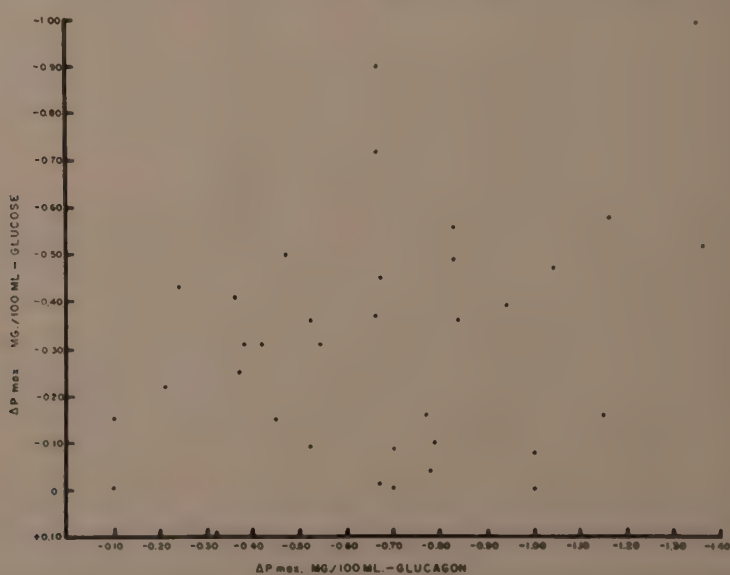

FIG. 3. Correlation graph between maximal serum inorganic phosphorus fall induced by dextrose and by glucagon.

serum inorganic phosphorus, independent from that determined by secondary insulin liberation. Speculatively, it may be thought that this effect depends upon the utilization of phosphate in the disintegration of glycogen by phosphorylase.

Summary. The effect of intravenous glucose upon serum inorganic phosphorus was compared to that of glucagon in 35 diabetic patients taken at random.

The subjects were classified in two groups according to their serum phosphate fall following glucose. In a group of 19 patients, the phosphate fall after glucose was less than 0.30 mg/100 ml, and in another group of 16 patients, more than 0.30 mg/100 ml similar to that found in non-diabetic individuals. As a working hypothesis, it has been assumed that diabetics with a normal phosphate fall are producing insulin. It was found that the group with a normal phosphate fall after glucose showed a greater fall after glucagon than the group with defective phosphate fall. On the other hand, the group with defective or absent phosphate fall after glucose also showed a very definite fall after glucagon. It would seem then that the latter substance exerts a direct effect upon serum inorganic phosphate, independent of the reactive insulin secretion produced by the glucose load. This effect is possibly related to the activation of phosphorylase which acts in the process of disintegration of glycogen through the introduction of phosphate in the bonds existing between the hexose molecules.

1. Kirtley, W. R., Waife, S. O., and Peck, F. B., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 387.

2. Kirtley, W. R., Waife, S. O., Helmer, O. M., and Peck, F. B., *Diabetes*, 1953, v2, 345.

3. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

4. Fiske, C. H., and Subbarow, Y., *ibid.*, 1925, v66, 375.

5. De Venanzi, F., and Masin, F., *Acta Cientif. Venezolana*, 1950, v2, 214.

6. De Venanzi, F., *ibid.*, 1952, v3, 214.

7. De Venanzi, F., Roche, M., and Vera, J., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 16.

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Anti-Inflammatory Activity of Δ^1-9α-Fluorohydrocortisone Acetate. (21956)

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There have been many important advances in the use of steroids as a method of treatment of the inflammatory diseases during the past few years. Since the introduction of hydrocortisone as one of the most efficacious treatments, it has been continually modified with the hopes of increasing its antiphlogistic activity while diminishing its undesirable side effects. It has been found that halogenation at the C-9 position greatly increases the glycogen deposition potency(1,2) and the anti-inflammatory effects(3) but causes a relatively greater increase in mineralocorticoid activity (4,5,6). A second analogue of hydrocortisone, 1-dehydrohydrocortisone, has been reported to possess greater antiarthritic properties in man (7) than the parent compound. It has also been shown that this compound is a more potent glucocorticoid in animals(8,9) and that this increase is not associated with an increase in mineralocorticoid potency(8).

It was, therefore, of interest to study the effects of both of these modifications when introduced into the same molecule. Accordingly, the anti-inflammatory activity of Δ^1-9α-fluorohydrocortisone acetate was compared with that of hydrocortisone. Data are also included showing the relative potencies of Δ^1-hydrocortisone acetate, 9 α-fluorohydrocortisone acetate, and hydrocortisone for comparative purposes.

Materials and methods. Animals. Male rats obtained from the Upjohn colony (Sprague-Dawley ancestry) were used throughout these experiments. The body weights ranged from 150-170 g. All animals

were adrenalectomized and allowed free access to Archer dog pellets and 1% sodium chloride drink. They were housed in small wire cages with 4 animals per cage. *Steroids.* The steroids were injected subcutaneously as a suspension in a vehicle containing 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol, and 0.9% sodium chloride. The suspensions were made by grinding the crystalline steroids plus vehicle in a ground glass tissue homogenizer. The compounds* used were: (1) 11 β, 17 α, 21-trihydroxy - 4 - pregnene - 3,20 - dione (hydrocortisone), (2) 9 α-fluoro-11 β, 17 α, 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (9 α-fluorohydrocortisone acetate), (3) 11 β, 17 α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione, 21-acetate (Δ^1-hydrocortisone acetate), and (4) 9 α-fluoro-11 β, 17 α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione, 21-acetate (Δ^1-9 α-fluorohydrocortisone acetate). *Anti-Inflammatory Test.* The test used was a modification of the cotton pellet test as described by Meyer, *et al.*(11). It consisted essentially of implanting 2 nonsterile cotton dental pellets, weighing 6-8 mg, in the mid ventro-lateral subcutaneous connective tissue through a small incision in the skin which was closed by a metal wound clip. The implanting was done at the time of adrenalectomy while the animals were under anesthesia. Anesthesia was accomplished by intraperitoneal injection of sodium pentobarbital at a dosage of 40 mg per kg. Seven days after implantation, the pellets, with their accumulated granulation tissue, were dissected out, dried at 60°C for 24 hours, and weighed to the nearest 0.1 mg on a Roller-Smith Torsion balance. The original cotton weight was subtracted from the final value in order to get the net amount of granulation tissue. The injection of the compounds was begun at the time of implantation and continued through seven daily injections. The volume of each injection was 0.2 cc. The animals were sacrificed approximately 24 hours following the last injection. The experiments were designed so that each steroid was tested at two dosage levels in parallel with hydrocortisone which was used as the standard. The potency ratios were calculated by the method of Irwin(10) and are expressed in terms of activity of hydrocortisone, which is assigned a value of 1. The data on Δ^1-9 α-fluorohydrocortisone acetate were pooled from 3 separate experiments and the data on Δ^1-hydrocortisone acetate were pooled from 2 experiments.

Results. The comparison of the antiphlogistic potency of Δ^1-hydrocortisone acetate, 9 α-fluorohydrocortisone acetate, and Δ^1-9 α-fluorohydrocortisone acetate with hydrocortisone is summarized in Table I. These data show that the potency ratio for Δ^1-hydrocortisone acetate is 3.1, for 9 α-fluorohydrocortisone acetate 7.3, and for Δ^1-9 α-fluorohydrocortisone 14, whereas hydrocortisone is assigned a value of 1.

Discussion. It has been reported(7) that Δ^1-hydrocortisone is 2 to 3 times as effective as hydrocortisone in the treatment of arthritis. Others(8,12) have found that it is 3.0 to 3.9 times as active as hydrocortisone by the liver glycogen deposition test. The anti-inflammatory test, as described here, shows that Δ^1-hydrocortisone acetate is 3.1 times as active as the parent compound. These results, therefore, are in good agreement with the clinical data and results on glycogen deposition.

The activity of 9 α-fluorohydrocortisone acetate has been found to be 12 times hydrocortisone by the glycogen deposition test(12) and 13 times hydrocortisone acetate by an anti-inflammatory test similar to the one described in this paper(3). It is interesting to note that the data reported here show that this halogenated compound is 7.3 times as active as hydrocortisone.

It is seen by examination of the data in Table I that Δ^1-9 α-fluorohydrocortisone acetate is 14 times as effective as hydrocortisone. It was found by Stafford, *et al.*(12) that it was approximately 50 times as active as hydrocortisone on glycogen deposition. Therefore, an apparent quantitative discrepancy exists between the classical glucocorticoid activity and antiphlogistic effect. This could be due to at least two possible reasons: (1) glycogen deposition and anti-inflammatory ef-

* The compounds used in this study were kindly supplied by the Departments of Biochemistry and Chemistry, Research Division, The Upjohn Co.

TABLE I. Anti-Inflammatory Assays of Hydrocortisone, 9α-Fluorohydrocortisone Acetate, Δ^1-Hydrocortisone Acetate and Δ^1-9α-Fluorohydrocortisone Acetate.

Treatment	Dose	No. rats	Mean dry granuloma wt (mg) ± S.E.	Potency (× hydrocortisone)
CMC	0.2 cc	20	14.41 ± .77	
Hydrocortisone	200 μg	17	9.97 ± .46	
	1000 "	16	6.57 ± .34	1
Δ^1-9α-Fluorohydrocortisone acetate	10 "	20	9.91 ± .46	
	50 "	19	7.90 ± .47	14
CMC	0.2 cc	7	13.40 ± .47	
Hydrocortisone	200 μg	7	9.80 ±1.25	
	1000 "	6	6.32 ± .29	1
9α-Fluorohydrocortisone acetate	25 "	7	10.74 ± .34	
	125 "	8	6.05 ± .44	7.3
CMC	0.2 cc	10	12.20 ± .49	
Hydrocortisone	200 μg	8	10.10 ± .65	
	1000 "	9	5.70 ± .42	1
Δ^1-Hydrocortisone acetate	60 "	10	10.00 ± .77	
	300 "	10	6.20 ± .50	3.1

fects are mediated through different mechanisms, or (2) the nature of the tests are such that differenecs in physical properties of the compounds, such as solubilities, could affect the results of the two tests in a quantitatively different manner.

The fact that Δ^1-9 α-fluorohydrocortisone acetate has an anti-inflammatory activity greater than the additive effects of Δ^1-hydrocortisone acetate and 9 α-fluorohydrocortisone acetate is of significance. The mechanism responsible for the increase in activity produced by introducing two important chemical changes into the same molecule is not clear at present but is worthy of further investigation.

Summary. A comparison of anti-inflammatory effects of hydrocortisone with several of its analogues has been made. It was found that Δ^1-hydrocortisone acetate was 3.1 times as active as hydrocortisone; 9 α-fluorohydrocortisone acetate was 7.3 times as active, while Δ^1-9 α-fluorohydrocortisone acetate was 14 times as effective as hydrocortisone, as determined by the cotton pellet implantation method.

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1. Fried, J., and Sabo, E., *J. Am. Chem. Soc*, 1953, v75, 2273.

2. ———, *ibid.*, 1954, v76, 1455.

3. Singer, F. M., and Borman, A., *Fed. Proc.*, 1955, v14, 281.

4. Borman, A., Singer, F. M., and Numerof, P., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 570.

5. Swingle, W. W., Baker, C., Eisler, M., LeBrie, S. J., and Brannick, L. F., *ibid.*, 1955, v88, 193.

6. Liddle, G. W., Pechet, M. M., and Bartter, F. C., *Science*, 1954, v128, 496.

7. Bunim, J. J., and Pechet, M. M., *J.A.M.A.*, 1955, v157, 311.

8. Perlman, P. L., and Tolksdorf, S., *Fed. Proc.*, 1955, v14, 377.

9. Herzog, H. L., Nobile, A., Tolksdorf, S., Charney, W., Hershberg, E. B., and Perlman, P. L., *Science*, 1955, v121, 176.

10. Irwin, J. O., *Suppl. J. Royal Stat. Soc.*, 1937, vIV, 1.

11. Meyer, R. K., Stucki, J. C., and Aulsebrook, K. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 624.

12. Stafford, R. O., Barnes, L. E., Bowman, B. J., and Meinzinger, M. M., *ibid.*, 1955, v89, 371.

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Fluorescent Antibody and Complement-Fixation Tests of Agents Isolated In Tissue Culture from Measles Patients.* (21957)

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Enders and Peebles(1) observed that human or monkey kidney tissue cultures inoculated with specimens from measles patients undergo, after one or more passages, characteristic nuclear and other cytologic changes. Both infectious virus and specific complement-fixation antigen appeared concurrently in the cultures and the cytopathogenic effect was neutralized by convalescent-phase measles serum. We have applied the fluorescent antibody technic of Coons and his colleagues(2,3) to similar cultures and find that the results parallel those of complement-fixation tests and thus provide immunochemical support for the data of Enders and Peebles and in addition evidence that measles antigen(s) are present in the nuclei as well as in the cytoplasm of infected cells.

Methods. Patients were selected for study during a mild outbreak of typical rubeola. Clinical diagnosis was aided in certain cases by recognition of giant cells of the Warthin-Finkeldey type in stained films of nasal discharge obtained early in illness, as described by Tompkins and Macaulay(4). *Collection of specimens and preparation of tissue cultures.* The procedures outlined by Enders and Peebles(1) were followed. Blood, throat swabs or washings, or nasal discharge were obtained before or within a day after the appearance of the rash. When possible, specimens were inoculated into tissue cultures within a few hours after their collection. Tissue cultures consisted of trypsinized monkey kidney cells grown in sheets(5). Tubes were inoculated with 0.2 or 0.3 ml of the prepared specimens, fed with nutrient fluid to bring the volume to 1.0 ml, and incubated as stationary slants at 36.5°C. Nutrient fluid consisted of beef amniotic fluid containing 5% inactivated horse serum and 5% beef embryo extract. The first passage contained 100 units of penicillin, 100 μg of streptomycin, and 50 μg of nystatin (fungicidin)(6); others, 50 units and 50 and 10 μg, respectively. Transfers to fresh tubes were carried out at 14-day intervals with 0.2-ml amounts of pooled fluids taken 5, 9, and 14 days after inoculation. Controls consisted of uninoculated cultures and of cultures passaged serially with fluids from uninoculated tubes. *Staining with fluorescein-labeled antibody.* The monkey kidney cells were gently scraped from the tube walls, or sedimented by centrifugation of the tissue culture fluid, washed with NaCl buffered at pH 7.6(7), sedimented, and spread on slides. The films were dried in air and fixed with acetone. They were often stored at 4°-6°C for from 1 to 3 days before staining by the indirect method of Weller and Coons(3). The test serum diluted 1:20 was acute- or convalescent-phase measles or heterologous human serum. Fluorescent antibody was prepared from antihuman gamma globulin horse serum generously supplied by Dr. David Gitlin, Children's Medical Center, Boston. The pseudoglobulin fraction was conjugated with fluorescein isocyanate, dialyzed, and stored at −20°C. Before use it was absorbed with mouse liver powder to remove nonspecific staining(2), then diluted 1:10. Prior to staining with the fluorescent antibody solution, films of uninoculated and inoculated preparations were treated, one of each with acute-, the other with convalescent-phase measles serum, to control the staining

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technics. The equipment used for fluorescence microscopy has been described by Buckley, Whitney, and Rapp(8). *Complement-fixation antigens* consisted of the fluid phase from tissue cultures in tubes or in 200-ml square bottles. For the latter, 1-ml amounts of inoculum and 7 ml of nutrient fluid were used. Medium 703(9) with or without 5% inactivated horse serum was substituted for Enders' medium(1) in some lots with satisfactory results. The fluids were centrifuged at about 2500 r. p.m. for 15 minutes and inactivated at 56°C for 30 minutes. *Complement-fixation tests* were made with three 50% units of complement. Fixation was at 4°C for 24 hours. For serum titrations, 2-fold dilutions were tested against a previously determined optimum dilution of antigen. Titers were the calculated number of 50% units of complement fixed by undiluted serum.

Results. Transmissible agents, presumably viruses, were isolated from both blood and a throat swab of one patient, and from nasal discharge and a throat swab, respectively, of 2 others whose blood was not obtained. The throat swab specimens had been stored in a dry ice chest for 7 days. No agents were detected in specimens from 5 additional cases. Inocula from 3 of the latter had been stored at 4°-6°C for 2 days before culture; tests of inocula from the other 2 were discontinued after 3 apparently negative serial passages. These circumstances may have contributed to our failure to isolate agents.

Enders and Peebles(1) and Rustigian *et al.* (10) encountered latent virus-like agents that induce marked vacuolization and syncytial masses in monkey kidney tissue cultures. The cellular degeneration characteristic of these "monkey-kidney agents" frequently appeared in our cultures, both in those inoculated with specimens from measles patients and in controls; hence cytologic criteria for recognition of measles agents were difficult to apply. In some tissue culture series the "monkey-kidney agents" destroyed the cell sheets in 10 to 14 days. We therefore relied on tests for the presence of measles antigen to identify the 3 agents cultivated from measles inocula.

The reactivity of the measles agents in both fluorescent antibody and complement-fixation tests seemed to be specific. Cells from control monkey kidney cultures, some with extensive "foamy" areas and multinucleated cells, did not exhibit fluorescence nor did control tissue culture fluids fix complement with either acute- or convalescent-phase sera from measles cases. Cells infected with measles agents, however, were brightly fluorescent with the 3 measles convalescent-phase sera tested but not with 3 acute-phase sera or with human antisera to herpes simplex, influenza Types A and B, or mumps viruses. The proportion of brightly and specifically fluorescent cells increased as serial passages continued. After 4 passages, many of the cells apparently contained measles antigen. One of the strains has been carried through 7 passages.

In tests of infected cells, fluorescence was observed in some cultures as early as the 5th day of incubation (Table I). Earlier tests were not made. Between the 5th and 10th day, fluorescence, when present, was usually confined to the cytoplasm. After about 2 weeks, however, bright yellow-green fluorescent intranuclear masses were frequently seen in fluorescent cells, very rarely in non-fluorescent cells (Fig. 1-3). The masses were surrounded by an unstained dark nuclear zone. Attempts to correlate the appearance of specific fluorescent masses in the nuclei with the

FIG. 1. Monkey kidney cells 14 days after infection with measles agent No. 5584. Indirect fluorescent antibody stain with convalescent-phase measles serum. The lighter areas are those showing specific yellow-green fluorescence. 400×.

FIG. 2. Monkey kidney cells 13 days after infection with measles agent No. 5585. Stain same as Fig. 1. 700×.

intranuclear inclusions described by Enders and Peebles are in progress.

In direct comparisons between development of specific fluorescence and of complement-fixation antigen, the fluorescent antibody procedure seemed the more sensitive of the two methods, since it detected antigen earlier in the course of incubation than did the complement-fixation test (Table I). This difference may have been due to the fact that fluorescent antibody tests were done on cell spreads, whereas complement-fixation antigens were made from centrifuged fluids. The fluid antigens were relatively weak; they rarely were active when diluted more than twofold. The antigen titer of one fluid taken 25 days after inoculation was considerably increased by addition of an extract of cells from the same cultures. The cells were frozen and thawed for 6 cycles in NaCl solution buffered at pH 7.6; the supernate was the extract.

FIG. 3. Control for Fig. 2; same cells stained with acute-phase measles serum. 700×.

Only 3 pairs of acute- and convalescent-phase sera could be obtained from measles patients. The complement-fixation titer of each acute-phase serum was <4; those of the convalescent-phase specimens were 40, 64, and 200. In tests with 21 sera from 13 cases of measles the distribution of titers agrees with that found by Enders(11) (Table II). Among the 26 heterologous sera tested were convalescent-phase sera from cases of virus infection such as herpes simplex, influenza, and mumps, and unclassified sera received for routine tests for evidence of virus or rickettsial infections. Twenty-three gave relatively low complement-fixation titers.

Discussion. Demonstration that tissue cultures inoculated with specimens from measles patients produce antigens that react specifically with convalescent-phase measles sera substantiates the findings of Enders and

TABLE I. Correlation of Results of Fluorescent Antibody Tests with Appearance of Complement-Fixation Antigen in Monkey Kidney Tissue Cultures Infected with Measles Agents.

Strain	Tissue culture		Fluorescent antibody results with		CF titer*
	Passage level	Day post-inoculation	Acute-phase serum	Convalescent-phase serum*	
5582, 5583† (blood, throat swab)	3	14	0	+	300
	4	9	0	+	NT
		14	0	+	211
	5	9	0	±	NT
		14	0	+	179
5584 (throat swab)	3	4	NT	NT	<4
		9	NT	NT	>64
		14	0	+	303
	4	10	NT	NT	<8
		14	0	+	<8
		18	NT	NT	<8
		21	0	+	112
	5	14	0	+	<4
	6	5	0	+	<4
		10	0	0	97
		14	0	+	55
		25	0	+	221
5585 (nasal discharge)	1	14	0	±‡	NT
	2	14	0	+	NT
	3	14	0, ±	+	NT
	4	13	±	+	NT
	5	5	0	+	<4
		10	0	0	<4
		14	0	+	<4
		25	0	+	195

CF = complement-fixation. NT = not tested.

Green-yellow fluorescence due to labeled antibody was graded as follows: 0 = none, ± = faint or rare cell bright, + = bright.

* Convalescent-phase serum 55291 used for both procedures.

† Combined blood (5582) and throat swab (5583) isolates from the same patient.

‡ Serum 5586 used in this test.

Peebles(1). We did not repeat their filtration experiments, but were unable to recover fungi, or bacteria including pleuropneumonia-like organisms and leptospira from infected tissue cultures and presume, therefore, that the antigens that produced specific reactions in fluorescent antibody and complement-fixation tests were derived from measles virus. On the whole, antigen in infected tissue cultures was detected earlier by the fluorescent antibody method than by complement-fixation tests. Preliminary data suggest, however, that the latter are of value in differential diagnosis of exanthemata and encephalitides of unknown origin.

The intranuclear fluorescence that appeared after prolonged incubation of infected cultures looks like that observed by Leduc, Coons, and Connolly(12). They made indirect fluorescent antibody stains of sections of lymph nodes from rabbits immunized with diphtheria toxoid and found spots of antibody within nuclear shadows that closely resemble the intranuclear measles antigen we noted (*cf.* their Fig. 4 and 5, our Fig. 1 and 2). Whether the intranuclear measles antigen is associated with inclusion bodies demonstrable by Giemsa or other stains(1) is still to be determined. One of us has recently observed that poliomyelitis virus antigen appears later in the nuclei than in the cytoplasm of monkey kidney cells under circumstances quite similar to those in the present study(13).

Summary. Agents isolated in monkey kidney tissue cultures from 3 cases of measles were identified by immunologic tests with convalescent-phase measles serum. Measles antigen was detected in infected cells by the in-

TABLE II. Complement-Fixation Titers of Sera with Measles Agent Tissue Fluid Antigens.

Titer range*	Sera from cases of measles: Days after onset† <5	>5	Heterologous sera
<4	7	0	7
5- 25	1	1	11
26- 50	0	1	5
51-100		3	2
101-200	1		1
>200		4‡	

* Titers = Number of 50% units of complement fixed.

† Exact date of onset unknown in some cases; may refer to earliest symptoms or appearance of rash.

‡ Three additional sera from one patient taken 3 to 4½ months after onset fell in this group.

direct fluorescein-labeled antibody technic before specific complement-fixation antigen appeared in the fluid phase. Specific fluorescence due to the presence of measles antigen was noted in the nuclei as well as in the cytoplasm of some cells from infected cultures.

1. Enders, J. F., and Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

2. Coons, A. H., and Kaplan, M. H., *J. Exp. Med*, 1950, v91, 1.

3. Weller, T. H., and Coons, A. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 789.

4. Tompkins, V., and Macaulay, J. C., *J.A.M.A.*, 1955, v157, 711.

5. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

6. Hazen, E. B., and Brown, R., *ibid.*, 1951, v76, 93.

7. Wadsworth, A. B., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 3rd ed., Williams and Wilkins, Baltimore, Md., 1947, p. 226.

8. Buckley, S. M., Whitney, E., and Rapp, F., PROC. SOC. EXP. BIOL. AND MED., in press.

9. Healy, G. M., Fisher, D. C., and Parker, R. C., *Canad. J. Biochem. and Physiol.*, 1954, v32, 327.

10. Rustigian, R., Johnston, P., and Reihart, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 8.

11. Enders, J. F., personal communication, August 30, 1955.

12. Leduc, E. H., Coons, A. H., and Connolly, J. M., *J. Exp. Med.*, 1955, v102, 61.

13. Buckley, S. M., *Arch. ges. Virusforsch.*, in press.

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Chemotherapy of Experimental Schistosomiasis. IV. Oral Activity of Antimony Trichloride Antibiotic Complexes. (21958)

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(Introduced by Nathan B. Eddy.)

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1 - (Diethylaminoethylamino) - 4 - methylthiaxanthone, Miracil D, was the first non-antimonial compound found to be of value for the oral treatment of schistosomiasis(1). In later studies, however, Miracil D proved to be toxic to man at therapeutic dosage levels(2,3). Other compounds which have been used with varying degrees of success in the oral treatment of schistosomiasis in man or experimental animals include antimony potassium tartrate, tartar emetic(4); sodium antimony III bis-pyrocatechol-2,4-disulfonate, Fuadin (5); various thioantimonials(6); and some symmetrical diaminodiphenoxyalkanes(7). During the course of screening compounds in this laboratory, it was found that the antimony trichloride complexes of oxytetracycline, Terramycin; of chlortetracycline, Aureomycin; and of tetracycline were schistosomacidal in white mice. The present report includes a comparison of the oral activities of these complexes and of other antimony compounds.

Materials and methods. Samples of the 3 complexes were synthesized by Dr. Philip N. Gordon and were submitted for evaluation by Chas. Pfizer & Co., Inc. The dithiastibiole compound mentioned below was supplied

TABLE I. Toxicity and Activity of Antimony Compounds.

Compound	Acute oral LD_{50}		Treatment results	
	Drug (mg/kg)	Sb equivalent (mg/kg)	No. mice	% reduction of worms by 10 oral doses totaling 350 mg Sb/kg
Terramycin antimony trichloride	1450	174	35	53.5
Aureomycin antimony trichloride	1550	248	35	83.6
Tetracycline antimony trichloride	1110	187	24	91.0
Fuadin	>1200	>162	15	0
Tartar emetic	540	198	18	94.3
Antimony trichloride	510	272	14	81.2
4,5-Dihydro-2-hydroxy-4-methylol-1,3,2-dithiastibiole	>1575	>732	9	0

through the courtesy of Abbott Laboratories. The drugs were given orally to white Swiss mice. In addition, the complexes were tested by the subcutaneous and intraperitoneal routes. After the compounds were found to be schistosomacidal by the preliminary method of screening described by Luttermoser(8), further evaluation of their activities was carried out as follows. Commencing on the 35th day after exposure to about 300 *Schistosoma mansoni* cercariae, mice in groups of 5 or 6 each were treated orally twice daily with the same dose of antimony in the form of one of the different compounds for a period of 5 days. Two to 3 weeks after treatment, the animals were killed and the schistosomes living in the portal system were perfused out by the technic of Yolles *et al.*(9) and counted. Small pieces (1 cm^2) of the livers of the treated mice were pressed between glass slides and examined for dead worms trapped in lesions. Unless dead worms were found, no reduction in worm infection was claimed. The worms living in the untreated mice were likewise isolated and counted by the same procedure. The percentage of worms remaining alive in the treated groups was inferred from the total number of living worms recovered from the treated mice relative to the number obtained from the untreated mice. From these data the percentage reduction in infection resulting from treatment was calculated. The LD_{50} values were determined on mice by the method of Litchfield and Wilcoxon(10).

Results. Table I summarizes the data on the activity and toxicity of the various compounds. The minimum oral dose of the Terramycin antimony trichloride complex which cleared the mice of more than one-half of their parasites was about 300 mg/kg (equivalent to about 35 mg antimony/kg) of body weight, while the oral LD_{50} of this compound for uninfected mice was about 1450 mg/kg(11). Fuadin, one of the drugs of reference, was not schistosomacidal at oral doses as high as 425 mg/kg. The curative results obtained with the first complex given orally at a level equivalent to 35 mg antimony/kg twice daily for 5 days were compared with those obtained by giving the same oral dose of antimony in the form of tartar emetic, antimony trichloride, and 4,5-dihydro-2-hydroxy-4-methylol-1, 3, 2-dithiastibiole. The Terramycin complex was more active than the last-named compound, which was reported by Schubert(6) to be among the most active schistosomacidal compounds when fed to mice at higher levels than used in this study. In comparison with tartar emetic and with antimony trichloride, the Terramycin antimony complex demonstrated no advantage in activity at this dosage. When Terramycin and antimony trichloride in quantities equivalent to the dosage of the complex were given separately but one following the other, reduction of live parasites equal to that obtained by administration of the Terramycin antimony complex was observed. Oral regimens of Terramycin, Aureomycin, or tetracycline alone were not schistosomacidal at oral doses comparable to the amount of these antibiotics in the complexes. Preliminary comparison of the activities and toxicities of the Terramycin antimony complex with the Aureomycin and tetracycline complexes (Table I) indicates that the last 2 are more active than the first but have similar toxicities.

Thus the apparent therapeutic advantage of the Aureomycin antimony complex or the tetracycline antimony complex over the Terramycin antimony complex is questionable. The 3 antibiotic antimony complexes were active following subcutaneous or intraperitoneal injection, but were toxic at doses of 50 mg/kg and higher.

Discussion. It appears probable that the schistosomacidal activity and toxicity of the 3 antibiotic complexes result almost entirely from their antimony content, and that variations in the efficacy of these compounds compared with the other antimony-containing preparations mentioned arise from differences in solubility, absorption, and dissociation. Such variations in activity further emphasize the need for additional investigation of organic antimonial compounds which are schistosomacidal upon oral administration.

Summary. The antimony trichloride complexes of Terramycin, Aureomycin, and tetracycline were active orally against *S. mansoni* infections in white Swiss mice. These compounds were no more active than tartar emetic or antimony trichloride.

1. Kikuth, W., Gonnert, R., and Mauss, H., *Naturwiss.*, 1946, v33, 253.

2. Halawani, A., *J. Roy. Egyptian M. A.*, 1949, v32, 29.

3. da Silva, J. R., *Rev. brasil. med.*, 1953, v9, 577.

4. Walker, J., *Ann. Soc. belge de méd. trop.*, 1928, v8, 273.

5. El Ayadi, S. M., *J. Roy. Egyptian M. A.*, 1947, v30, 562.

6. Schubert, M., *Am. J. Trop. Med.*, 1948, v30, 525.

7. Raison, C. G., and Standen, O. D., *Brit. J. Pharmacol.*, 1955, v10, 191.

8. Luttermoser, G. W., *J. Parasitol.*, 1954, v40, 130.

9. Yolles, T. K., Moore, D. V., DeGiusti, D. L., Ripsom, C. A., and Meleney, H. E., *ibid.*, 1947, v33, 419.

10. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. and Exp. Therap.*, 1949, v96, 99.

11. Personal communication from Dr. J. E. Lynch, Pfizer Therapeutic Institute.

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Effect of Liver Damage and Nephrectomy on Convulsant Potency of Pentylenetetrazol (Metrazol)* (21959)

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There is lack of agreement on the fate and excretion of pentylenetetrazol (Metrazol) in laboratory animals and man. On the basis of data obtained by *chemical procedures,* Tatum and Kozelka(1) concluded that Metrazol is detoxified by the liver in dogs and man, whereas Esser and Kuehn(2) reported that the major portion of the drug is excreted by the kidney in man. Recent studies in our laboratory(3) with radioactive Metrazol indicate that most of a given dose of the drug in rats is excreted by the kidney in an altered form. On the basis of data obtained by *biological procedures,* Dille and Seeberg(4) concluded that Metrazol is degraded by the liver in cats, whereas Voss(5) reported that the drug is largely excreted by the kidney in rats. In view of these conflicting reports, it was thought important to make a systematic study of the effect of liver damage and of nephrectomy on the convulsant activity of this agent. It was anticipated that such a study would contribute information on the relative role of the liver and the kidney in the detoxification and excretion of Metrazol. The results obtained provide the basis for this report.

Methods. Adult male albino mice obtained from the Carworth Farms (CF #1) and adult male albino rats obtained from the Holtzman-Rolfsmeyer Farms were used as experimental

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animals. They were maintained on Purina Laboratory Chow and allowed free access to food and water except during the actual experimental procedure. The convulsant potency of *intravenously* administered Metrazol† was determined in control, liver-damaged, and nephrectomized mice; the convulsant potency of *subcutaneously* administered Metrazol was determined in control, liver-damaged, nephrectomized, and sham-operated mice and rats. In the determination of the convulsant potency, groups of at least 8 animals were given various doses of the drug until at least 3 points were established between the dose required to convulse 16% of animals and that to convulse 84% of animals. The results obtained were then plotted on logarithmic probability paper and a regression line visually fitted to the plotted points. From this plot of the data, the dose of Metrazol which was convulsant to 50% of animals (CD_{50}) was determined and the 95% confidence limits were calculated by the method of Litchfield and Wilcoxon(6). Intravenously, the drug (0.5% aqueous solution) was injected rapidly into the dorsal tail vein of the mouse; the endpoint employed was either a minimal seizure (any overt clonic activity which persisted for at least 5 seconds), or a full maximal seizure as indicated by the presence of the hindleg tonic extensor component(7). Subcutaneously, the drug (0.5% aqueous solution in mice, 5.0% in rats) was injected into a loose fold of skin over the neck; the endpoint employed was the occurrence of a minimal seizure, as described above, within one hour after the injection. Liver damage in mice was produced by a modification of the method of Stowell and Lee (8), *i.e.*, by the oral administration of 4 ml/kg of a 40% (w/v) solution of carbon tetrachloride (CCl_4) in olive oil. Maximum hepatic damage occurs about 100 hours after such administration and therefore Metrazol was tested at this time. Liver damage in rats was induced by a single subcutaneous injection of 2 ml/kg of a 50% solution (w/v) of CCl_4 in peanut oil. Histological studies of livers taken from rats treated in this manner have shown that maximum central and midzonal necrosis occurred approximately 48 hours after the injection of CCl_4; accordingly, Metrazol was tested at this time. Bilateral nephrectomy was performed in both species by the retroperitoneal approach while the animals were under light ether anesthesia. In order to keep the effect of accumulated metabolic products relatively constant, Metrazol was tested 6 and 12 hours after nephrectomy in mice and rats, respectively. Sham operation was performed by the same technic as for nephrectomy, except that the kidneys were not disturbed.

† Kindly supplied by Dr. C. L. Moench, Bilhuber-Knoll Corp.

TABLE I. Effects of Liver Damage and of Nephrectomy in Mice on Convulsant Potency of Intravenously Administered Pentylenetetrazol (Metrazol).

Exp. procedure	Convulsant potency (CD_{50}), mg/kg (intrav.) Max seizures	Min seizures
Control	29.0 (27.1-31.0)	21.0 (19.3-22.9)
Liver damage	29.0 (26.8-31.4)	19.5 (17.4-21.8)
Nephrectomy	28.5 (27.0-30.1)	24.5 (22.7-26.5)

Values in parentheses represent 95% confidence limits.

Results. The effects of liver damage and of nephrectomy in mice on the convulsant potency of intravenously administered pentylenetetrazol (Metrazol) are shown in Table I. The CD_{50}s for *intravenous* Metrazol for *maximal* seizures determined in liver-damaged mice and nephrectomized mice were not significantly different from the CD_{50} determined in control animals. On the other hand, the CD_{50} for *intravenous* Metrazol for *minimal* seizures, a more sensitive test, tended to be lower in liver-damaged mice and higher in nephrectomized mice. In view of these observations, it was decided to determine the CD_{50} of Metrazol administered by a route which would provide a longer time interval before the onset of convulsions than that provided by the intravenous route. It was anticipated that the longer sojourn of Metrazol in the body would induce more marked differences in the CD_{50}s determined in liver-damaged and nephrectomized animals, and thus delineate the relative

TABLE II. Effect of Liver Damage and of Nephrectomy in Mice and Rats on Convulsant Potency of Subcutaneously Administered Pentylenetetrazol (Metrazol).

	Convulsive potency (CD_{50}), mg/kg s.c. route—min seizures	
Exp. procedure	Mice	Rats
Control	59.0 (55.2-63.1)	47.0 (43.9-50.3)
Liver damage	46.5 (43.3-50.0)	39.5 (36.4-42.9)
Nephrectomy	66.0 (59.5-73.3)	45.5 (41.9-49.6)
Sham operation	65.0 (59.6-70.9)	42.0 (38.7-45.6)

Values in parentheses represent 95% confidence limits.

importance of these organs in the fate and excretion of the drug. Accordingly, Metrazol was injected *subcutaneously* in mice and rats and the CD_{50} for *minimal* seizures determined. The results obtained are summarized in Table II. The CD_{50}s for subcutaneous Metrazol are significantly lower in liver-damaged mice (21%) and rats (18%) than the control CD_{50}s. In sharp contrast, the CD_{50}s for subcutaneous Metrazol in nephrectomized mice and rats were not significantly different from the CD_{50}s in control or sham-operated animals. These data suggest that the liver plays the major role in the metabolic degradation of Metrazol in both species.

Discussion. The fact that CCl_4-induced liver damage and nephrectomy in mice had no significant effect on the intravenous CD_{50} of Metrazol is easily explained. The rapid intravenous injection of Metrazol elicits seizures so quickly that the organs concerned with the detoxification and excretion of this drug have only seconds in which to exert an effect. Hence, any influence which the liver or the kidney may exert is largely circumvented.

Liver damage lowered the CD_{50} for subcutaneously administered Metrazol by 21% in mice and 18% in rats. This suggests that the liver is important for the degradation of this drug. Additional evidence in support of this conclusion is the fact that fatalities after Metrazol were more frequent in liver-damaged than in normal mice. For example, in the dose range of 40 to 47 mg/kg, subcutaneous Metrazol was fatal to 37.5% of liver-damaged mice, whereas all normal mice survived the drug even in the higher range of 50 to 66 mg/kg. These observations are in agreement with the evidence that Metrazol is converted into an inactive product and that the site of this conversion is the liver(9).

Since there was no significant difference in the CD_{50}s of subcutaneously administered Metrazol determined in control, nephrectomized, and sham-operated mice and rats, it would appear that, within the time interval required to absorb the drug after subcutaneous injection, no appreciable amount of Metrazol is excreted by the kidney. This interpretation is in agreement with the observation of Esplin *et al.*(3) that less than 1% of intravenously administered radioactive (C^{14}) Metrazol is excreted by the rat kidney as an inactive product during the first 30 minutes, and that a total of 75% is excreted in an inactive form over a 48-hour period. Thus, the kidney appears to play a relatively minor role in the excretion of Metrazol or of an active conversion product.

Summary. The convulsant potency of intravenously administered Metrazol for maximal and minimal seizures was determined in control, liver-damaged, and nephrectomized mice. In addition, the convulsant potency of subcutaneously administered Metrazol for minimal seizures was determined in control, liver-damaged, nephrectomized, and sham-operated mice and rats. The results obtained were analyzed to determine the relative importance of the liver and the kidney in the fate and excretion of the drug. Neither liver damage nor nephrectomy had any significant effect on the potency of intravenously administered Metrazol for maximal or minimal seizures. Liver damage significantly increased the potency of subcutaneously administered Metrazol for minimal seizures; on the other hand, bilateral nephrectomy and sham operation had no significant effect on the potency of this drug. The data indicate that, in both mice and rats, the liver is the principal organ for the conversion of Metrazol into products devoid of convulsant activity, and that, within the time required for the complete absorption of subcutaneously injected Metra-

zol, the kidney plays no important role in the metabolic alteration or excretion of this compound.

1. Tatum, H. J., and Kozelka, F. L., *J. Pharmacol. and Exp. Therap.*, 1941, v72, 284.

2. Esser, A., and Kuehn, A., *Deutsche Z. ges. gerichtl. Med.*, 1933, v21, 474.

3. Esplin, D. W., Woodbury, D. M., and Goodman, L. S., *Fed. Proc.*, 1954, v13, 352.

4. Dille, J. M., and Seeberg, V. P., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 624.

5. Voss, J., *Arch. f. exp. Path. u. Pharmakol.*, 1926, v118, 259.

6. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. and Exp. Therap.*, 1949, v96, 99.

7. Goodman, L. S., Grewal, M. Singh, Brown, W. C., and Swinyard, E. A., *ibid.*, 1953, v108, 168.

8. Stowell, R. E., and Lee, C. S., *Arch. Path.*, 1950, v50, 519.

9. Esplin, D. W., unpublished observations, 1954.

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Biological Method for Determination of Glucocorticoid Activity of Crystalline Compounds and Urine Extracts.* (21960)

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During the past decade, several biological methods for assay of compounds exhibiting glucocorticoid activity have been established. These technics have been applied to the assay of urine extracts to measure excretion levels of adrenal cortical hormone by the human. The methods of Reinecke and Kendall(1), Olson, Jacobs, Richert, Thayer, Kopp, and Wade(2), and Pabst, Sheppard, and Kuizenga (3), are based on capacity of glucocorticoids to deposit glycogen in livers of adrenalectomized rats after glycogen stores have been depleted by fasting. These methods use male rats weighing 140 to 180 g and injections of test material are made at regular intervals throughout the assay. The methods are reasonably specific for assay of glucocorticoids and reproducible with a fairly high degree of precision. The disadvantage of the methods lies in their comparative lack of sensitivity. Lack of sensitivity was apparently overcome by using the mouse as assay animal. The method of Venning, Kazmin, and Bell(4) measures deposition of glycogen in depleted livers of fasting adrenalectomized mice, while those of Eggleston, Johnston and Dobriner (5), and of Dorfman, Ross and Shipley(6) measure capacity of adrenal corticoids to prevent loss of hepatic glycogen during fasting. Our experience with the method of Venning, Kazmin and Bell demonstrated the assay to be unreliable with strains of mice available to us. Nissim(7), who conducted a critical study of various methods, believes that precision obtained by Venning *et al.* is dependent upon selection and careful control of a particularly suitable strain of mice bred in their own laboratories.

Existing methods were not suitable for our needs. The methods using young adult rats were not sensitive enough for assay of urine extracts, and uneconomical from the standpoint of animal cost and amount of test material necessary for assay. The mouse methods offered economy both in animals and test materials, but the strains of mice generally available did not lend precision to the assay. It therefore became desirable to develop a method based on satisfactory features of other methods. The method we devised is presented in this communication.

Materials and methods. The animals were 21-day-old male rats of the Holtzman strain weighing 50 to 55 g. These animals were

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placed in a cage, given Rockland Rat Diet and tap water *ad libitum*, to become accustomed to laboratory conditions for a period of 2 days. After this the animals were bilaterally adrenalectomized and returned to the cage. Animals had access to a solution of 5% cerelose in 0.9% NaCl as drinking water until the following morning, then the solution was replaced with one containing 0.9% NaCl only. A good supply of food was available at all times until the evening of the second post-operative day. The animals were then deprived of food. On the morning of the third post-operative day, saline drinking solution was removed, the test compounds injected subcutaneously, and each animal placed in an individual quart Mason jar containing about 2 inches of sawdust. The jars were covered with sealer rings fitted with hardware cloth mesh. It is extremely important that rats be isolated from one another at this point. Occasionally an animal of the control group, or one given an inactive preparation, becames hypoglycemic and subject to convulsions. Such animals tend to fight with other rats if they are caged together. Test materials were administered in 0.1 cc of corn oil per rat. Cortisone was first dissolved in acetone, an aliquot then transferred to corn oil so that the desired dosage was contained in 0.1 cc, and acetone evaporated by vacuum. Six hours after injection of test material, the animals were individually weighed, sacrificed by cervical dislocation, the entire liver quickly removed and transferred to a hot 30% solution of KOH for digestion. A 50 cc pyrex, round-bottom centrifuge tube containing 10 cc of KOH solution proved to be very satisfactory. Small funnels were placed in top of the tubes to serve as condensers and the tubes heated in boiling water bath just previous to autopsy. Digestion process was complete in 30 or 40 minutes, after which the glycogen was precipitated by adding absolute ethyl alcohol (about 1.2 times the volume of the digest) while the digests were still hot. The digests were then brought to a boil, cooled in a cold water bath, again brought to a boil and re-cooled as suggested by Pabst, Sheppard and Kuizenga(3). The KOH-liver digests were placed in refrigerator for at least 2 hours to insure more complete precipitation and coagulation of the glycogen. If necessary, the liver digests may be refrigerated over night. After this, the samples were centrifuged at 2500 rpm for 10 minutes, the supernatant decanted and discarded, and the tubes inverted on absorbent paper and allowed to drain. The precipitated glycogen was then washed with a small amount of absolute alcohol, centrifuged, and again allowed to drain. Glycogen was hydrolyzed by adding 6 cc of 0.6N HCl and placing the tubes in an autoclave at 15 lb pressure for 20 minutes. After cooling to room temperature the hydrolysates were neutralized with 10% NaOH using Phenol Red as an indicator. The solutions were centrifuged, and glucose determined by the method of Reinecke(8). The values obtained were then expressed as glucose per 100 g body weight per rat.

Results: Determination of time for autopsy. In the initial phases it was important to determine the most favorable time at which animals should be sacrificed after injection of cortisone, which was to be employed as the standard. Groups of animals were killed at 2, 4, 6, 8 and 10 hours after injection and amount of liver glycogen deposited was determined. The cortisone in this experiment was 100 μg, a dose which preliminary study indicated would give a good response and yet be somewhat less than maximal. The results of this study are shown in Table I. During the first 2-hour period after injection of cortisone there was no indication of glycogen deposition over and above the level found in oil-treated control animals. At 4 hours the animals had deposited a highly significant amount of gly-

TABLE I. Amount of Liver Glycogen Deposited at 2-Hr Intervals after 100 μg Cortisone Injection.

Interval, hr	No. animals	Glycogen,* avg ± S.E., mg	Increase, mg	"t"
0				
2	7	.66 ± .046		
4	9	10.14 ± .58	9.473	14.013†
6	8	20.25 ±1.52	10.11	6.46 †
8	9	29.02 ±1.46	8.77	4.16 †
10	7	22.76 ±5.25	−6.26	
Oil controls at 6 hr	6	.68 ± .074		

* Glycogen expressed as glucose/100 g body wt.
† P = <0.01.

TABLE II. Glycogen Deposition in Response to Various Doses of Cortisone.

Dose cortisone, μg	No. animals	Glycogen, mg Avg	S.E.	C,* %	"t"
0	25	.68	.074	54	
10	12	2.52	.329	45	7.99‡
30	13	9.69	.925	34	7.1 ‡
50	17	13.8	1.08	32	2.79‡
100	18	22.64	1.07	20	4.9 ‡
200	18	28.67	2.24	33	2.43†
300	19	39.35	1.74	19	3.75‡

* Coefficient of variation; expresses stand. dev. as % of mean response. Avg "C" for all cortisone doses is 30.5%.

† P = <.05.

‡ P = <.01.

Glycogen expressed as mg glucose/100 g body wt.

cogen. Expressed as mg glucose/100 g rat weight, a level of 10.14 mg was found. At 6 hours and again at 8 hours post-injection, highly significant increases in liver glycogen were noted. During the 2-hour period from 6 to 8 hours, deposition of glycogen was somewhat less than that observed during the preceding 2-hour period, and at 10 hours post-injection, the peak glycogen deposition in response to a single injection of cortisone had been passed and glycogen was again being mobilized. It was then decided that 6 hours post-injection was the optimum time for sacrificing the animals. During the period between 2 and 8 hours post-injection, the deposition of liver glycogen progressed as a linear function of time. After 8 hours the concentration of glycogen in the liver decreased. It was considered preferable to select a time during which glycogen was being deposited at a steady rate and before the peak was reached.

Development of a dose-response curve. Cortisone was employed as the standard hormone in development of the assay method, and the data in Table II show that a satisfactory dose-response relationship exists over a relatively large range of doses. Highly significant differences in glycogen deposition were obtained with 20 μg increments in dosage of cortisone in the lower dosage range. A measurable and highly significant response was obtained with as little as 10 μg of cortisone. These results indicate that the assay is much more sensitive than any of the existing methods utilizing the 140 to 180 g rat. In the method suggested by Pabst, *et al.*, a dose of 350 μg of cortisone was necessary to deposit a significant amount of glycogen in the liver, and dose increments of 30 μg were necessary to elicit significant increases in liver glycogen. The *coefficient of variation*(9) has been calculated for each dosage and the average coefficient of variation for cortisone is shown in Table II. Averages for the methods of Olson, *et al.* and Pabst, *et al.*, as calculated by Nissim(7), are 29.5% and 23.25% respectively. The average reported here is 30.5% indicating only slightly higher variation in these animals. The method of Venning (using her strain of mice) is perhaps more sensitive to smaller changes in dosage of cortisone than the method suggested here. She has shown a significant change in glycogen deposition with as small an increment as 10 μg of cortisone, but does not report any liver glycogen values for smaller doses. The 10 μg dose is also in the lower limit for a significant response in our method.

The data in Table II have been used to plot the dose-response curve found in Fig. 1. The mean values of glucose per 100 g weight were plotted against the log.-dose of cortisone and the curve obtained indicated a straight line relationship. From these values the slope,

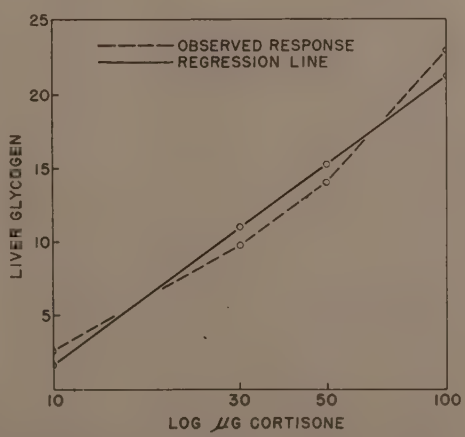

FIG. 1. The mean values for liver glycogen expressed as mg of glucose per 100 g body wt, plotted against the log-dose of cortisone.

"b", which was calculated according to the formula $\frac{\Sigma xy}{\Sigma x^2}$ as discussed by Snedecor(9), was found to be 19.559. The regression line was then calculated according to the formula $\hat{Y} = \bar{y} + b(X - \bar{x})$. The index of precision was calculated according to the formula $\lambda = s/b$ wherein "s" is equal to the average standard deviation about the curve and "b" is the slope of the regression line. According to Emmens(10) the value of λ is a guide to the precision of the assay; a minimal value for λ indicates a steep regression line in relation to magnitude of variance.

It is evident from Table II that as the dose increases above 100 μg of cortisone, there is a sharp rise in the standard error of the mean. Precision indices were calculated using the data from doses a) 0 to 300 μg of cortisone; b) 0 to 200 μg of cortisone; and c) 0 to 100 μg of cortisone. The indices were found to be 0.225, 0.273, and 0.183, respectively. Accepting the fact that for a precise bioassay the value for lambda should be minimal, it is apparent that the range of doses from 0 to 100 μg of cortisone gives the most precise information.

Assay of glucocorticoids in urine. The primary purpose for the development of this assay was to establish a method by which levels of glucocorticoid material could be measured in human urine. Chloroform extracts of urine were prepared according to Mason's modification(11) of an original method for chemical determination of corticoids by Daughaday, Jaffe and Williams(12). Some difficulty was encountered using the crude chloroform extracts of normal urines. Attempts to show a dose-response relationship with normal urine were unsuccessful at the outset. Response to increasing doses of urine extracts was not a linear increase in liver glycogen. In many cases the results showed a decrease in amount of glycogen as the dose of urine extract was increased. This pattern of results suggested that either there was some active principle in urine extracts that was inhibiting glucocorticoid activity, or that there was inactive material, poorly soluble in the volume of oil used, that was interfering with absorption of the glucocorticoids. Many of the urine extracts possessed large amounts of color, all of which appeared to be readily soluble in any of the non-polar solvents. In view of the behavior of crystalline steroids in a benzene-water partition system, as shown by Talbot, Saltzman, Wixom and Wolfe(13), it was believed that this treatment might be beneficial in purifying urine extracts. Talbot, *et al.* found that if the active corticoid compounds were dissolved in benzene and partitioned with water, the only compound that remained in the benzene to any extent was desoxycorticosterone. The more highly oxygenated steroids preferentially moved into the water phase. This procedure was applied to urine extracts and it was found that the pigmented material remained almost entirely in the benzene fraction and that essentially all materials active in liver glycogen deposition moved into the water phase. The experimental data are presented in Table III.

TABLE III. Effect of Benzene-Water Partition of Urine Extracts on Glycogen Deposition.

Extract	Dose equivalent, hr	Glycogen crude extract, mg	Glycogen benz. fract., mg	Glycogen HOH fract., mg
A	3	.646		1.45
	6	1.27	.706	2.15
B	2	30.28	1.62	32.14

Glycogen expressed as mg glucose/100 g body wt.

Benzene-water partitioning considerably reduced the amount of solid material in urine extracts, giving a cleaner preparation that could be dissolved in a small volume of oil for injection. There is some indication that higher levels of glycogen deposition are obtained with the same hourly equivalents of urine extract after a benzene-water partition.

The data obtained from the assay of urine extracts from normal male individuals is shown in Table IV. A dose-response relationship is indicated which shows a highly significant change in glycogen deposition between 200 and 400 ml urine equivalent doses, and a significant change between 400 and 600 ml dose levels. These data are expressed graphically in Fig. 2 together with the standard curve for cortisone for comparison. Re-

TABLE IV. Glycogen Deposition in Response to Doses of Urine Extract from Normal Individuals.

Dose equivalent, ml	No. animals	Glycogen,* mg Avg	S.E.	"t"	Cortisone† equivalent, μg	Cortisone equiv./l, μg
200	6	1.24	.37		9.6	48.0
400	5	6.31	1.18	4.43§	17.5	43.75
600	6	9.09	.51	2.30‡	24.0	40.0

* Glycogen expressed as glucose/100 g body wt. † Determined from stand. curve; Fig. 1.
‡ P = <.05. § P = <.01.

gression lines for cortisone and for urine extract, the slope of which was 16.553, were tested statistically to see if they deviated significantly from parallel. The value "t" was calculated according to the formula: $t = \frac{b_1 - b_2}{\sqrt{\sigma_{d^2}}}$. The value "$b_1 - b_2$" is equal to the difference between slopes and the quantity $\sqrt{\sigma_{d^2}}$ is the standard error of the difference between regression coefficients(14). The value for "t" was 1.633 which showed the two regression lines did not deviate from parallel significantly. Therefore, it can be assumed that urine extracts may be assayed in terms of cortisone. When calculated as μg equivalents of cortisone excreted per liter of urine, as shown in Table IV, it is clear that fairly close agreement in results exists between the dose levels administered.

A comparison of the values for corticoid excretion obtained by this method with determinations made by other investigators also show close agreement. This method shows an average excretion of 44 μg cortisone/l of pooled normal male urine. Schneider(15) has obtained a value of 53.1 μg/l, Zaffaroni (16) reports a range of 20 to 40 μg/l, and Venning(17) reports values of 40 to 80 μg/l of urine of normal individuals. Determinations of Schneider and of Zaffaroni were by means of paper chromatography and Venning's values by biological assay.

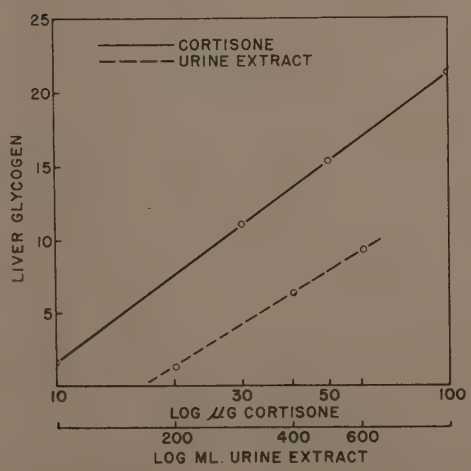

FIG. 2. Regression line for liver glycogen response to urine extracts compared with the regression line for response to cortisone.

Validity of the assay procedure for measurement of variations in corticoid excretion is demonstrated by an experiment wherein a patient was given ACTH intravenously for periods of 3 days interspersed with 3-day control periods. Urine was collected, extracted, and assayed for glucocorticoids by the bioassay method reported here. Table V shows glucocorticoid, and the formaldehydogenic corticoid excretion in response to ACTH stimulation. Results showing an increase in excretion of glucocorticoids during ACTH administration, indicate that this method measures secretion products of the adrenal cortex which influence carbohydrate metabolism, and that the method is sufficiently sensitive to detect fluctuations in excretion of glycogenic steroids in urine.

In Table V the results of both assays are expressed as μg of cortisone excreted per 24 hours. It can be seen that by chemical assay the excretion level is considerably higher than that shown by biological determination. This indicates that chemical determination is measuring formaldehydogenic compounds which do not have glycogenic activity. In the calculation of corticoid excretion the assumption is made that 1 mole of formaldehyde is derived from 1 mole of formaldehydogenic ster-

TABLE V. Daily Corticoid Excretion in Urine during ACTH Stimulation.

Treatment*	Glucocorticoids, μg equiv. "E"/24 hr	FGS,† μg "E"/24 hr
Control	91.2	931.
5 mg ACTH	2380.	3870.
Control	120.	1167.
10 mg ACTH	1298.	3794.
Control	266.	675.
25 mg ACTH	1440.	4125.

* Consecutive 3-day periods.
† Formaldehydogenic steroids.

oid. This method of calculation does not make allowance for differences in glycogen-depositing activity of various formaldehydogenic steroids. It is well known that the formaldehydogenic method measures biologically inactive metabolites of glycogenic steroids as well as active forms. Since the isolation of pregnance-3, 17, 21-triol 11, 20-dione (tetrahydro E) in 1951 by Schneider(15), much investigation into excretion of corticoid metabolites has been conducted.

The introduction of -glucuronidase by Kinsella, Doisy and Glick(18) for hydrolysis of urinary steroids greatly increased the yield of chemically detectable corticoids. Interesting studies on urine and blood levels of corticoids and their metabolites have recently been reported by Baggett, Kuisella and Doisy(19) and Silber and Porter(20). These investigators have also found increased yields of urinary corticoids by enzymatic hydrolysis and, in addition, Silber and Porter showed that the main corticoid in blood is hydrocortisone (Compound F) while in urine it is tetrahydro F, 94% of which is excreted as a glucuronide. Studies by Fukushima, Leeds, Bradlow, Kritchevsky, Stokem and Gallagher, (21) have disclosed 4 new metabolites excreted in urine following ACTH administration. These compounds, designated by the authors as "cortol, -cortol, cortolone, and -cortolone," are not reducing substances and are negative to the Porter-Silber reaction, however, they do yield formaldehyde on periodic acid oxidation.

These increased yields of urinary corticoids and newly discovered metabolites tend to further the understanding of adrenal function and metabolism of cortical secretions to a degree that could not have been arrived at by means of a biological assay alone. It has, however, been shown by Venning(22), Lobotsky, Hannye and Lloyd(23), and Chart(24), that for the routine bioassay of urinary corticoids the hydrolysis of urine with -glucuronidase does not add greatly to the information obtainable by acid hydrolysis. The great increase in yield of chemically detectable corticoids is not reflected by a comparable increase of activity in biological assay procedures. This fact does not negate the usefulness of bioassay procedures or of the enzymatic hydrolysis of urine for determination of corticoid excretion in urine. It is of importance to establish the relative activity of newly isolated metabolites and to quantitate the potency of new synthetic steroids as they become available.

We believe that this bioassay procedure can function as a valuable adjunct to the chemical methods for the evaluation of corticoid excretion as well as being an accurate, simple and economical method for determination of glycogen-depositing activity of crystalline compounds.

Summary. A practical bioassay technic for glucocorticoids is proposed, using the adrenalectomized, 21-day-old male rat as test animal. The method offers the accuracy and precision shown by the use of the young adult rat, and the sensitivity and economy observed in methods using the laboratory mouse. Dose-response curves have been established for cortisone and for the assay of urine extracts. The regression lines for the two substances are parallel, indicating the method is valid for measurement of glucocorticoid excretion. It is suggested that this bioassay method may serve as a valuable adjunct to the chemical methods for the assay of urinary corticoids.

1. Reinecke, Roger M., and Kendall, Edward C., *Endocrinology*, 1942, v31, 573.

2. Olson, R. E., Jacobs, F. A., Richert, D., Thayer, S. A., Kopp, L. J., and Wade, N. J., *ibid.*, 1944, v35, 430.

3. Pabst, M. L., Sheppard, R., and Kuizenga, M. H., *ibid.*, 1947, v41, 55.

4. Venning, E. H., Kazmin, V. E., and Bell, J. C., *ibid.*, 1946, v38, 79.

5. Eggleston, N. M., Johnston, B. J., and Dobriner,

K., *ibid.*, 1946, v38, 197.

6. Dorfman, R. I.; Ross, E., and Shipley, R. A., *ibid.*, 1946, v38, 178.

7. Nissim, J. A., *ibid.*, 1953, v52, 611.

8. Reinecke, Roger M., *J. Biol. Chem.*, 1942, v143, 351.

9. Snedecor, George W., *Statistical Methods*, 4th ed., Iowa State College Press, Ames, Iowa, 1946.

10. Emmens, C. W., *Hormone Assay*, Academic Press, Inc., New York, 1950.

11. Mason, H. L., personal communication.

12. Daughaday, W. H., Jaffe, H., and Williams, R. H., *J. Clin. Endocrinol.*, 1948, v8, 166.

13. Talbot, N. B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K., *J. Biol. Chem.*, 1945, v160, 535.

14. Fisher, R. A., *Statistical Methods for Research Workers*, G. E. Stechert & Co., New York, 1941.

15. Schneider. John J., *J. Biol. Chem.*, 1952, v194, 337.

16. Zaffaroni. A. Burton, R. B., and Keutmann, E. H., *Science*, 1950, v6, 11.

17. Venning, E. H., and Kazmin, V. E., *Endocrinology*, 1946, v39, 131.

18. Kinsella, R. A., Jr., Doisy, R. J., and Glick, J. H., Jr., *Fed. Proc.*, 1950, v9, 190.

19. Bagget, Billy, Kinsella, R. A., and Doisy, E. M., *J. Biol. Chem.*, 1953, v203, 1013.

20. Silber, R. H., and Porter, C. C., *ibid.*, 1954, v210, 923.

21. Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B., and Gallagher, T. F., *ibid.*, 1955, v212, 449.

22. Venning, E. H., *Fed. Proc.*, 1952, v11, 302.

23. Lobotsky, J., Hannye, J. B., and Lloyd, C. W., Abstract No. 124, Program, Endocrine Society, Atlantic City, June, 1955.

24. Chart, J. J., Ph. D. Thesis, University of Wisconsin, 1954.

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Colorimetric Study of Absorption and Excretion of 4-(p-Dimethylaminostyryl) Quinoline Methiodide and Related Compounds.* (21961)

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Since 4-(p-dimethylaminostyryl) quinoline methiodide(1) and several related dyes, when administered in the diet, have been found to cause regression of Lymphoma 8 tumors in rats(2-4), the fate of the unchanged dye was studied colorimetrically. The results of this study have led to finding a compound that appears to be unusually active in bringing about regression of Lymphoma 8(3,4).

Methods. The compounds used were prepared in the chemical laboratories of Carson-Newman College by Wilfred Lyke and Joan Wilson, with the financial assistance of a Frederick Gardner Cottrell Grant from the Research Corporation and a grant from the Medical Research Foundation: 4-(p-Dimethylaminostyryl) quinoline methiodide (4M 2M). 4-(p-Dimethylaminostyryl) quinoline methochloride (4M2MCl). 2-(p-Diethylaminostyryl) quinoline ethiodide (2E2E). 2-(p-Dimethylaminostyryl) pyridine methiodide (2M2PM)‡, and 2-(p-Dimethylaminoanil) pyridine methiodide (2M2NPM). The usual method of administering the dyes was to mix them with powdered food of animals, by grinding in a ball mill 8 hours or longer. After offering dyed food for 2 days the animals were given untreated food *ad libitum*, beginning the evening of second day. In a few instances a solution of a dye in a 0.9% NaCl or 5% glucose was administered by a stomach tube or was injected subcutaneously in the flank or intravenously via a tail vein. Urine and feces were collected separately, by conventional apparatus, until 2 days after last dose of dye was administered. The dye was

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* Under contract with the U. S. Atomic Energy Commission.

‡ This compound was included because of the observation by workers at the Midwest Research Institute that it seemed to cause damage to Sarcoma 180 in mice used in tumor necrosis screening tests, although it did not cause conspicuous inhibition of the growth of tumors. Goodson, Lewis H., personal communication.

recovered from feces by hot 95% ethanol in a Soxhlet extractor and measured by Beckman Type B spectrophotometer. The absorption peak at 540 mμ was used for quinolinium compounds and the 469 mμ peak was used for 2M2PM. Absorption due to normal fecal pigment was taken into account. To measure the dye content of a tissue, such as liver, the sample was washed quickly with alcohol, then minced under a small amount of alcohol. If the pink color of dye appeared in the alcohol, the dye was extracted with the aid of a Soxhlet extractor. A sensitive test for traces of dye in urine and other aqueous solutions was carried out by cutting a thin wick from a disc of filter paper(5) and allowing the solution to rise through this wick and spread through the entire filter paper. Any 4M2MCl, or 2E2E would color the wick. Free dye was removed from urine by passing it through a tube packed with absorbent cotton or by stirring the sample with filter paper. After the dyed cellulose was washed with water and dried, the dye was eluted with boiling alcohol and the quantity measured colorimetrically. In some instances the decolorized urine gradually turned purple on standing, and could be recovered and measured as described. The appearance of pink or purple color could be hastened by heating the sample in a boiling water bath for about 20 minutes, provided the sample was slightly basic. Before heating, therefore, a base was added to urine samples that were acid. Appearance of pink color when a basic decolorized solution was heated, is referred to in this paper as a "heat-pink" test.

Results. Fecal excretion. From 36 to 47% of the 4M2M fed, was recovered from feces of Wistar rats, hooded rats, black mice, and brown mice. Practically the same results were obtained whether this dye was administered at concentration of 0.3% or 0.1% in Rockland Mouse Ration or concentration of 0.3% in starch, but a higher excretion was noted when food and dye were ground only 45 minutes. Recovery of 4M2MCl from the feces was about 58%, whereas recovery of 2E2E and 2M2PM was 13 to 27%. No 2M2NPM was recovered, perhaps because of hydrolysis in the stomach. The anil pyridines are usually readily hydrolyzed by acids, in contrast to the styryl compounds.

Disappearance of dye. The color of dye sometimes persisted 2 to 5 days at sites of subcutaneous injections of 4M2MCl solution in rats. This is in contrast with permanent staining reported to result from subcutaneous injections of 2-(p-acetaminostyryl)-6-(p-acetamidobenzamido) quinoline methoacetate (Styryl 430), reported to produce sarcomata (6). When rats were sacrificed after several days of eating food containing styryl dyes, their tissues did not appear to be stained. However, when a mouse was killed 20 minutes after receiving the last of three 1 ml oral doses of a solution containing approximately 3 mg of 4M2MCl/ml of 5% glucose at 20-minute intervals, 0.8% of the dye was recovered by extraction from the liver, and small quantity was extracted from kidneys. Similar results were obtained at the end of 3 hours, but no dye was found in kidneys and liver of 2 mice and a rat killed 1 day after receiving the solution by stomach tube. The color of saturated solution of 4M2M in 0.9% NaCl became lighter as it passed down the digestive tract of a rat or mouse, until it was yellow in the small intestine.§ This yellow solution brought about bleaching of dilute solutions of 4M2M or 2E2E within 4 hours. Liquid from the stomach did not produce this effect, nor did liquid from stomach, gall bladder, or intestine of a dog.

Urinary excretion. Rarely a faint trace of dye was observed in urine of animals fed dyed food, but stronger traces were noted following subcutaneous injections. When a rat ate 24.3 mg of 4M2MCl in his food during 2 days, only 0.033 mg of the free dye was found in urine of the first 24 hours and 0.023 mg in the next 24 hours. The additional dye recovered after decolorized urine had stood, was 0.05 mg for each day. After subcutaneous injection of 0.6 mg of 4M2MCl in 5% glucose, 5% of the dye was found in urine. The substance responsible for the color observed after the decolorized dye had stood for some time, had

§ This experiment was planned and executed with the assistance of Dr. Robert A. Woodbury, University of Tennessee, Memphis.

very nearly the same absorption maximum in alcohol as that of a known sample of 4M2M. A negative heat-pink test was obtained with urine of rats, rabbits, hamsters, and a dog that received subcutaneous injections of 4M2MCl solution, but the test was positive after eating of food containing 4M2MCl. The inference that the substance responsible for the heat-pink test was formed in the digestive tract was supported by further observations: A faint pink color was produced when the heat-pink test was applied to samples of 4M2M solution in 0.9% NaCl that had been decolorized in the digestive tract of a rat. A sample of 4M2M solution in 0.9% NaCl, kept over night at room temperature, after addition of a few drops of the liquid from the lower small intestine, then decolorized by passing through cotton, gave a strong heat-pink test. The nature of the substance responsible for the heat-pink test is being investigated further.

Summary. 1. A substantial portion, usually less than half, of the 4M2M, 4M2MCl, 2E2E, and 2M2PM administered to rats and mice in the diet, was found by colorimetric measurements, in the feces. 2. Only a trace of dye color was detected in urine of rats that had received 4M2M or 4M2MCl orally or subcutaneously. 3. Heating, or prolonged exposure to air, of samples of urine from animals that received 4M2M, 4M2MCl or 2E2E orally, not subcutaneously, produced a pink color. The trace of material responsible for this color seemed to be formed from the dye in the digestive tract. 4. Very little unchanged dye was found in the tissues of rats and mice that had received 4M2M or 4M2MCl orally. 5. A large part of the 4M2M or 4M2MCl administered orally is evidently converted into other substances.

The assistance of Edgar Cress and John Rafter in carrying out the animal experiments is acknowledged with appreciation.

1. Bahner, C. T., Pace, E. S., and Prevost, R., *J. Am. Chem. Soc.*, 1951, v73, 3407.

2. Hughes, B., Bates, A. L., Bahner, C. T., and Lewis, M. R., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 230.

3. Lewis, M. R., Hughes, B., Bates, A. L., and Bahner, C. T., *Growth*, 1955, v19, 1.

4. Bahner, C. T., accepted for publication in *Cancer Research*.

5. Saifer, A., Oreskes, I., *Anal. Chem.*, 1953, v25, 1539.

6. Browning, C. H., Gulbransen, R., and Niven, J. S. F., *J. Path. Bact.*, 1936, v42, 155.

7. Sheehan, H. L., *ibid.*, 1932, v35, 589.

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Tissue Abnormalities in Newborn Rats from Vitamin B_{12} Deficient Mothers. (21962)

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Reproductive failure in female rats deficient in vit. B_{12} has been reported by Dryden, Hartman and Cary(1), Lepkovsky *et al.* (2), Richardson, Witten and Couch(3). Thirty to 60% of the offspring died within 3 days and those which survived were underweight and unhealthy. Ferguson and Couch (4) observed degeneration in heart, liver, thyroid and kidney in chick embryos from hens deficient in B_{12}. This suggested that similar changes might be found in rat embryos and might account for their early mortality.

Methods. Weanling female rats were placed on B_{12} low diet of O'Dell *et al.*(5) until 4 weeks before mating when they were transferred to experimental diets. Basal diet was synthetic and contained 25% commercial soybean protein* extracted 6 times by boiling

* The Drackett soybean protein C-1, the Drackett Co., Cincinnati.

Tissues from newborn rats born to vit. B_{12} deficient mothers compared to those derived from control litters.

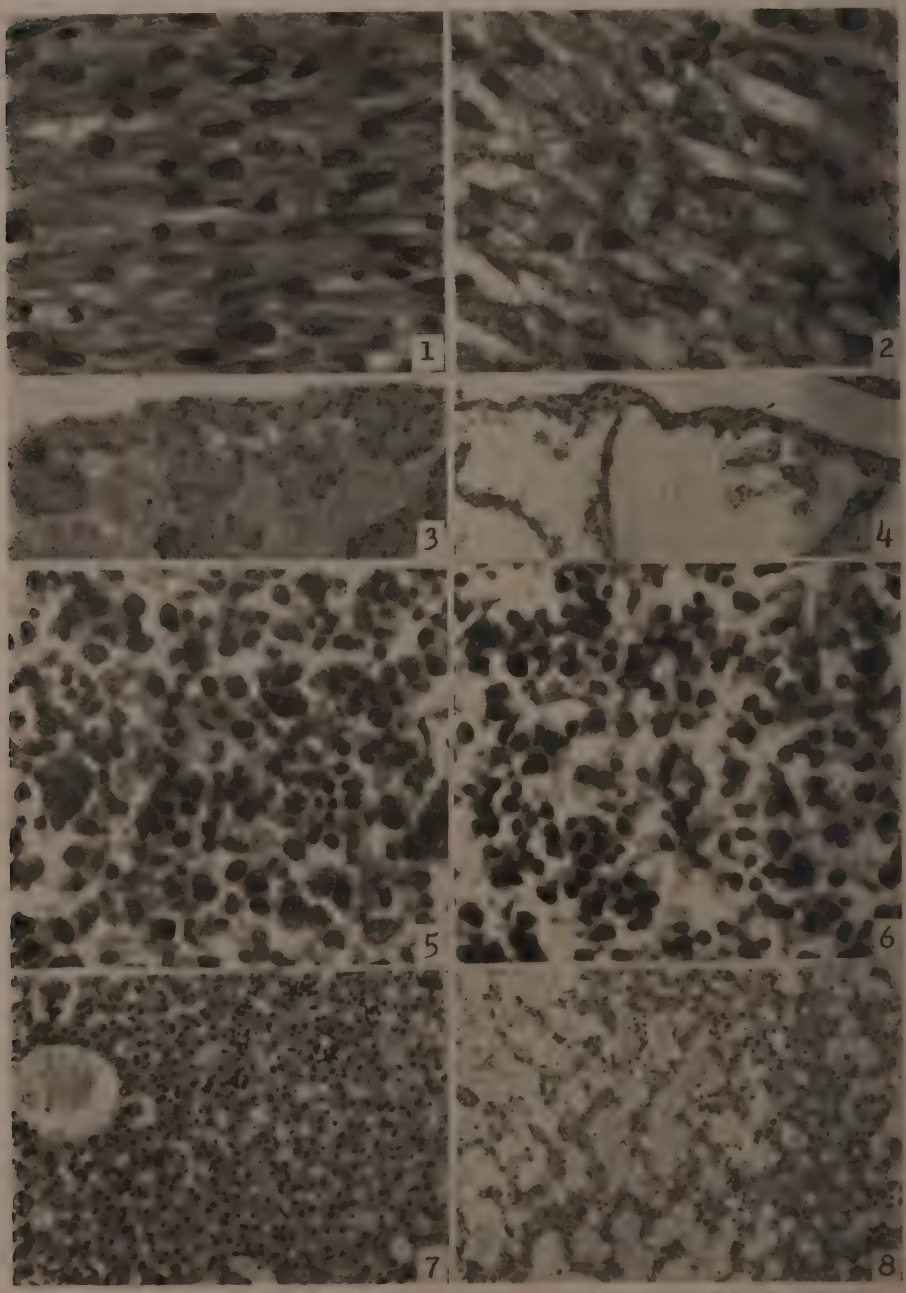

FIG. 1. Longitudinal section of heart muscle from control rat weighing 7.4 g. Chromatic nuclei and basophil cytoplasm. × 720.

FIG. 2. Longitudinal section of heart muscle from experimental rat weighing 2.7 g. Less chromatic nuclei, less differentiated cytoplasm and cell arrangement. × 720.
FIG. 3. Thick wall of auricular appendage from control rat. × 450.
FIG. 4. Very thin auricular wall from experimental rat of 2.7 g. × 450.
FIG. 5. Spleen of control rat with reticular cell background. × 720.
FIG. 6. Spleen of experimental rat (wt 4.15 g) lacking reticular primordial cells and filled with dense pyknotic nuclei with little cytoplasm. Population density of cells is low. × 720.
FIG. 7. Control liver with narrow uniform sinusoids basophil liver cells and numerous blood islands. × 450.
FIG. 8. Liver from experimental rat of 4.7 g showing dilated sinusoids filled with ghost red cells and fat. Note lack of basophilia and diminished size of liver cells. × 450.

70% isopropyl alcohol. One group received basal diet alone. The control group received a supplement of 25 μg of vit. B_{12}/kg basal diet. The vitamin-deficient females weaned only 40-70% of their young while controls weaned 95%. The young within 3 to 4 hours of birth were killed by ether and injected and fixed whole in either Bouin's solution or neutral formalin, or a mixture of 1 part formalin to 9 parts alcohol for study of glycogen. Ninety young from deficient mothers and 22 from control mothers were used. Hearts, livers, kidneys and spleens were excised, weighed and studied histologically in 7 μ paraffin sections. Other portions were sectioned at 13 μ in freezing microtome. Qualitative histochemical staining tests were used as follows: lipoids; Sudan IV on frozen sections; amyloid; dahlia or methyl violet(6); glycogen; Bauer-Feulgen; ribonucleic acid; toluidin blue with enzyme treated controls; desoxyribonucleic acid; methyl green pyronin(7).

Results. Structure and histology of organs of B_{12} deficient young. Experimental newborn rats were underweight, average 4.75 g (2.14-5.18) while control rats averaged 6.0 g (4.3-8.0). The gestation period for both was 21 days. Weight of heart, liver and kidney of experimental rats showed that whereas the former were below normal weight, they were heavier than normal in proportion to the rest of the body. The above are characteristic of immature animals.

Heart. Aortic arches showed no anomalies, although the chambers of the heart were not examined. The structure of muscle cells in both auricle and ventricle showed a fetal pattern, with little basophil cytoplasm, poorly developed myofibrillae and striations, and marked cell borders (Fig. 1, 2). Sudan staining showed small lipoid vacuoles in the cytoplasm. A few lymphocytes were seen in connective tissues. The figures compare longitudinal sections of ventricular muscle photographed under identical conditions. Nuclei were larger but less chromatic with a perinuclear ring of chromatin. D.N.A. determinations showed less nucleoprotein. Auricles were thin, not because of distension but because of failure to develop full thickness of wall (Fig. 3, 4).

Spleen. The control spleen showed a background of reticular cells against which small lymphocytes were contrasted. In the deficient animals the reticular cells had generally disappeared leaving a reticular net of fibers among which were many large, very dense nuclei, often pyknotic. These cells had little cytoplasm. Some cells with more cytoplasm contained hemosiderin (Fig. 5, 6).

Liver. In contrast to the fairly uniform sinusoidal pattern of normal liver, the liver of deficient young under low power showed a dilation of sinusoids between periphery and central veins. The dilated peripheral portions of the sinusoids contained normal non-nucleated red cells. Then followed a zone of red cell ghosts and fragments. The more central portions were plugged with fat globules of undetermined origin. Finally, the zone immediately around the central vein was packed with various leucocytic cells (Fig. 7, 8). This blockage of liver circulation resulted also in shrinkage of liver cords, disappearance of glycogen and nuclei with reduced D.N.A. Von Kupffer cells were all enlarged and contained much blood pigment. Hemopoietic islands of the liver were still present, but the cells, like those of the spleen, had large, dense or even pyknotic nuclei with little cytoplasm. (Fig. 9, 10, 11).

Kidney. The kidney of experimental animals showed retardation of growth more than any other organ. The cortex was of fetal

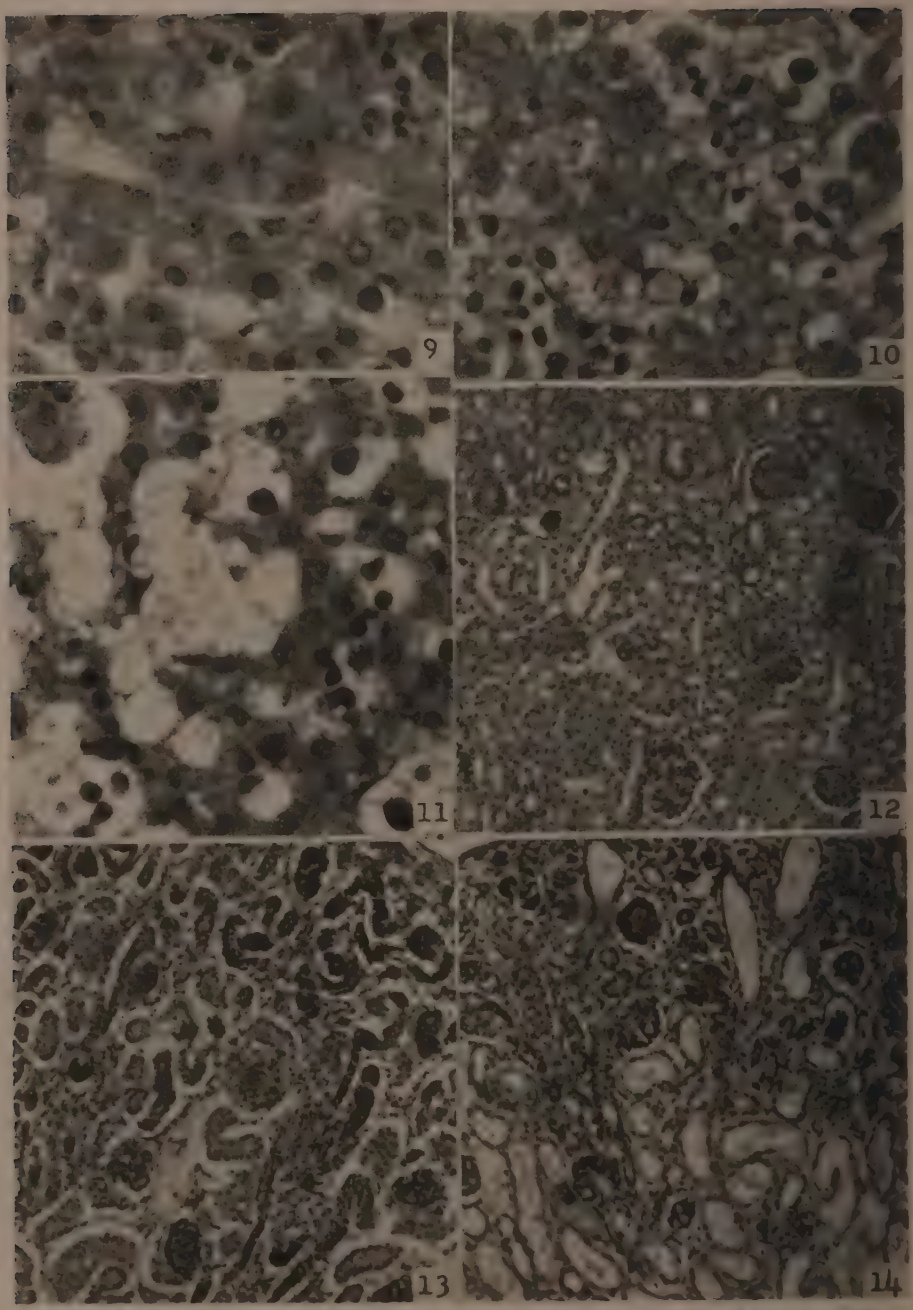

FIG. 9. Control specimen of liver with variable but chromatic nuclei and abundant cytoplasm. × 720.

FIG. 10. Liver from experimental animal of 2.7 g with loss of chromaticity in liver cell nu-

clei, loss of cytoplasmic volume and basophilia and transformation of both blood islands and cells of von Kupffer into pyknotic cells. × 720.

FIG. 11. Liver from deficient rat of 4.2 g wt with great dilation of sinusoids filled with fat and fragmented red cells and fading out of many liver cell nuclei. × 720.

FIG. 12. Cortex from kidney of a control rat (wt 7.4 g) showing one row of undifferentiated glomeruli and tubules. × 450.

FIG. 13. Kidney of deficient rat of 2.7 g showing several laminae of undifferentiated glomeruli and tubules and approximately 2 functional rows deeply placed. Lumens are not clearly defined. × 450.

FIG. 14. Kidney cortex from a deficient rat of 4.05 g showing great dilation of differentiated tubules and reduction of brush border or failure to develop such a border. × 450.

structure filled with incompletely formed glomeruli and tubules. The smallest animals showed the most immature picture (Fig. 12, 13, 14). In addition to the fetal picture, the convoluted tubules of young rats weighing about 4 g showed great dilation of tubules (Fig. 14), the capillary ducts and pelvis of the kidney. The brush border had either sloughed or had never formed. The lumina contained no detritus. Bowman's capsules also were dilated but were still functional. The tubules all resembled distal convoluted tubules. Sudan staining showed fine basal lipoid vacuoles in the tubular cells not seen in controls. On the other hand glycogen was lacking. Vascular extravasation was present together with extratubular edema. Newborn young weighing about 2.7 g showed a much less developed kidney in which no lumen was present in the tubules except in deeper, more mature and functioning units (Fig. 13). Here also was much extratubular fluid. In all of these abnormal kidneys there was abundant medullary mesenchyme as in the fetus but no amyloid deposits.

Discussion. It is a general fact that female rats placed on a low B_{12} diet show little evidence of abnormality in their tissues(8,9) but do develop deficiencies in their reproductive processes and severe growth deficiency in their young(1,3). The degree of growth deficiency and of pathology were coordinate with the immature birthweights, the smallest showing most severe effects. The work of many authors dealing with vit. A deficiency(10,11) with blocking agents like urethane, galactoflavin(12), methylene blue, irradiation(13), etc., show that whatever specificity these agents seem to have lies in the particular moment of susceptibility of tissues at the time of impact of the reagent or deprivation of some essential nutrient.

The case of B_{12} deficiency in addition to a possible correlation above showed that some of the results seen after birth were the indirect result of blocks in liver or kidneys. Similarly, cardiac anomalies were held responsible for urogenital hypoplasias in the work of Baird *et al.*(12) using galactoflavin.

There may be additional more specific effects in the present experiments because of the probable B_{12} requirement for synthesis of nucleoprotein(8,9). Hematological work on bone marrow and spleen is needed. Our sections showed great variability in cytoplasmic basophilia in erythrocytes. There was no anemia or general hypoxia in the mothers to indirectly affect the young.

Liver damage has been reported in swine (14) and in adult rat(9) as a result of feeding a B_{12} deficient diet. Ferguson and Couch(4) reported an increase of fat content in cells of heart muscle, in kidney tubules and in hepatic cells of chick embryos from hens on a diet low in vit. B_{12}. In addition, they reported damage to liver cells beyond this. These cell changes could be prevented by injection of B_{12} into the egg or into the deficient hens some time before the eggs were laid. The above seem to support the view of Ling and Chow(15,16) that this vitamin directly or indirectly plays a part in the accumulation and transformation of glycogen and fat.

The probable role of vit. B_{12} in protein metabolism and particularly in nucleoproteins was best seen in heart muscle. The cytoplasmic deficiency could have been due to overwork but was probably a part of the picture of immaturity. The failure to find any amyloid in connective tissues of the body also points to immaturity.

The abnormal changes in the heart, liver, kidney and spleen noted in our experiments with rats were entirely prevented by supple-

menting the deficient maternal diet with vit. B_{12}.

Conclusions. Female rats deprived of B_{12}, from before the time of mating until the end of gestation, produced deficient and weak progeny. This was shown in weights well below normal and arrested development proportionate to the subnormal weights. The defects involved vascular blocks especially in the liver with consequent passive congestion. The heart and kidneys were immature. Proximal convoluted tubules became blocked and transformed into distended tubules histologically like distal tubules. Spleens showed a loss of primitive reticular cells and transformation into large densely chromatic and pyknotic cells. The origin of the fat by which the liver sinusoids were blocked is not known.

1. Dryden, L. P., Hartman, A. M., and Cary, C. A., *J. Nutr.* 1951, v45, 337.

2. Lepkovsky, S. H., Boyson, H. J., Bouthilet, R., Penchary, R., Singman, D., Dimick, M. K., and Robbins, R., *Am. J. Physiol.*, 1951, v165, 79.

3. Richardson, L. R., Witten, P. W., and Couch, J. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 265.

4. Ferguson, T. M., and Couch, J. R., *J. Nutr.*, 1954, v56, 361.

5. O'Dell, B. L., Whiteley, J. R., and Hogan, A. G., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 349.

6. Pearse, A. G. E., *Histochemistry*, Little, Brown & Co., Boston, 1953.

7. Kurnick, N. B., *Stain Tech.*, 1952, v27, 233.

8. Stern, J. R., Taylor, M. W., and Russell, W. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 551.

9. Seigel, G. B., and Worley, L. G., *Anat. Rec.*, 1951, v111, 597.

10. Wilson, J. G., and Workany, J., *Am. J. Anat.*, 1948, v83, 357.

11. ———, *ibid.*, 1949, v85, 113.

12. Baird, C. D. E., Nelson, M. M., Monic, D. W., Wright, H. V., and Evans, H. M., *Fed. Proc.*, 1955, v14, 428.

13. Rugh, R., *Tr. N. Y. Acad. Sci.*, 1949, v12, 55.

14. Cartwright, C. E., Tatting, B., Robinson, J., Fellows, N. M., Gunn, F. D., and Wintrobe, M. N., Blood, *J. Hematol.*, 1951, v6, 867.

15. Ling, C. T., and Chow, B. F., *J. Biol. Chem.*, 1952, v198, 439.

16. ———, *ibid.*, 1954, v206, 797.

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Accumulation of Homologous Radioiodinated Albumin in Experimental Tumors. (21963)

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Heterologous proteins and amino acids have been shown to localize selectively in transplantable and spontaneous tumors of animals(1,2). This study was undertaken to determine whether homologous proteins are concentrated in tumor tissue since homologous albumin (human serum albumin) tagged with radioactive iodine is used extensively as a diagnostic tracer agent for the detection of brain tumors and metastatic lesions of the liver(3).

Several attempts to measure the distribution of radioactivity in biopsy specimens from tumors in humans have been inconclusive because of low activity in the tissues when permissible tracer doses were used. Accordingly, it was decided to study the problem in animals inoculated with experimental tumors, using homologous albumin.

Method. Rabbit albumin was obtained by ammonium sulfate separation, dialyzed and immediately lyophilized. Electrophoretic patterns indicated the material to be albumin, the single characteristic peak being visible. The albumin was trace iodinated with I^{131} according to the procedure of Gleason and Tabern (4), and yielded a protein of high specific activity (100-600 μc/ml with an albumin content of approximately 10 mg/ml). Ultracentrifugation and electrophoretic analysis have

TABLE I. Ratio of Radioactivity of Tumor and Invasive Zone to Normal Liver.

Rabbit No.			Day sacrificed	Counts/sec./g Normal liver	Invasive zone	Tumor	Invasive zone / Normal liver	Tumor / Normal liver
4	No Lugol's	Unperfused	1	5884	8461	12373	1.44	2.10
12	Lugol's	"	1	2938	4891	3661	1.66	1.25
6	No Lugol's	"	2	3191	4589	5535	1.44	1.73
26	Lugol's	"	3	3347	3174	9436	.94	2.82
16	"	"	5	474	618	720	1.30	1.52
						Avg	1.36	1.88
34	Lugol's	Perfused	1	711	894	1398	1.26	1.97
35	"	"	2	337	512	1816	1.52	5.39
30	"	"	3	680	748	2524	1.10	3.72
31	"	"	4	453	494	1745	1.09	3.85
						Avg	1.24	3.73

shown the end product to be similar to native albumin. The tagged albumin was injected intravenously into 9 albino and Dutch rabbits with actively growing Brown-Pearce tumors of the liver. The suspension of Brown-Pearce tumor was obtained by straining a thick homogenate through 2 layers of surgical gauze. Under ether anesthesia a laparotomy was done and about 0.2 ml of the tumor cell suspension was injected into the liver under direct vision. The rabbits were examined daily and after the tumor transplants had become palpable (5-23 days following inoculation), the iodinated albumin was injected intravenously in volumes of 1-5 ml. Some of the animals were given Lugol's solution in their drinking water in order to block their thyroid glands and prevent uptake of inorganic I^{131} from the injected albumin. The animals were divided into 2 groups: the animals of the first group were sacrificed without perfusion 1 to 5 days following the injection of the labelled albumin. They were killed by ether inhalation and autopsied immediately. The other animals were perfused 1 to 5 days following the injection of labelled albumin as follows: under ether anesthesia heparin was injected intraveounsly and a cannula was inserted into the right external jugular vein. Normal saline was injected into this cannula. Blood was drained through a cannula in the left common carotid artery until only saline was obtained. As soon as that happened, the thorax was opened and normal saline was injected into the descending aorta and drained through the inferior vena cava so as to perfuse the abdominal viscera. Tissue samples were then removed. Tumor tissue was carefully separated from normal liver tissue and sections through the "invasive" zone were taken. This "invasive" zone consisted of an area at the periphery of the tumor with equal amounts of tumor and normal liver tissue to each side of the center of the section. This was checked by histologic examination. The tissue samples were minced, blotted on filter paper and weighed into culture tubes. They were then dissolved in 2 ml 2N NaOH in a boiling water bath and, after they had cooled, were counted in a sodium iodide (thallium activated) scintillation well counter. The counts recorded were corrected for physical decay to the time of the administration of the dose.

Results. Table I shows the radioactivity expressed as counts/second/gram for normal liver, tumor and "invasive" zone tissue in Brown-Pearce inoculated animals. It can be seen that consistently higher activity is present in tumor tissue than in normal liver tissue. In the perfused animals, this difference appears to be somewhat greater.

Expressed as tumor/normal liver the ratios are 1.25 and 2.82 (average 1.88) in the unperfused animals and 1.97 to 5.39 (average 3.73) in the perfused animals. The ratio of "invasive" zone/normal liver in the unperfused animals ranged from 0.94 to 1.66 (average 1.36); in the perfused animals, it was 1.09 to 1.52 (average 1.24).

Scintigrams(5) showed concentration of radioactive iodine in the liver. There was not enough activity in the tumor, however, to ob-

tain satisfactory differentiation between normal liver and tumor with this method. This is evident from the ratios of tumor tissue/normal liver tissue.

The administration of Lugol's solution to some of the animals did not change the concentration of the iodinated albumin or the ratios between tumor, "invasive" zone and normal liver tissue (Table I).

Comment. Duran-Reynals(1) showed that heterologous proteins and dyes injected intravenously localize selectively in transplantable and spontaneous tumors of mice. He interpreted his findings as, "indicating that newly formed capillaries of tumors are, in general, more permeable than the capillaries of any normal tissue." In our animals, increased concentrations of homologous albumin were demonstrated in tumor tissue as compared to the surrounding normal liver. The ratio of specific activity of tumor/normal liver was even greater after the animals had been perfused. The reason for this may, indeed, be increased permeability of newly formed capillaries with escape of homologous albumin into tumor tissue. Once the albumin has escaped into tumor tissue, it cannot be removed by perfusion. Another possibility is actual metabolic utilization of homologous albumin by the more rapidly growing tumor tissue.

The labelled protein used in our study was homologous and presumably not denatured. It is assumed that it is handled by the rabbit like native albumin. Whether the intact protein molecule or a metabolite of this protein is deposited in tumor tissue cannot be stated at this time.

Conclusion. The data presented indicate that experimental tumors of the liver (Brown-Pearce) in rabbits accumulate more radioiodinated rabbit albumin than normal liver tissue of the same animal. This increased concentration appears to be unrelated to vascularity since it was demonstrated in perfused animals.

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1. Duran-Reynals, F., *Am. J. Cancer*, 1939, v35, 98.
2. Babson, A. L., and Winnick, Th., *Cancer Res.*, 1954, v14, 606.
3. Yuhl, E. T., Stirrett, L. A., and Libby, R. L., *Ann. Surg.*, 1953, v137, 184; Yuhl, E. T. and Stirrett, L. A., *ibid.*, 1953, v138, 857.
4. Gleason, G. I., and Tabern, D. L., personal communication.
5. Bauer, F. K., Goodwin, Wm. E., Libby, R. L., and Cassen, B., *J. Lab. and Clin. Med.*, 1952, v39, 153.

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Fecal Androgen, A Progesterone Metabolite in the Bovine.* (21964)

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Early investigations clearly indicated that pregnanediol is a product of metabolism of progesterone in man(1-4). Further *in vivo* studies have indicated that progesterone may be reduced to, and excreted as, allopregnanediol-3(α), 20(α)(5) and pregnanol-3(α)-one-20(6). However these metabolites in the urine account for only 5-20% of administered progesterone(7). Recent studies indicate that pregnanediol is not present in urine of pregnant goats(8) and cattle(9). The importance of a biliary pathway for elimination of progesterone metabolites has been indicated: (a), recovery of pregnanediol from bile of humans after progesterone was given parenterally (10); (b), identification of allopregnanediol-3(β), 20(β) in cattle bile(11); and (c), isolation of pregnanol-3(α)one-20 and pregnanediol-3(α), 20 (β) from bile of pregnant cows (12). It has been shown in studies of proges-

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terone-21-C^{14} metabolism in rats that most of the injected progesterone or its metabolites are carried to the intestine by bile and eliminated in the feces(13). In a study of steroid metabolism in the bovine, we previously determined the neutral ketonic non-alcoholic character of androgens in cow feces(14). Biliary androgenic activity has also been detected in this species(15). However, feces from intact or spayed heifer, following treatment with testosterone, contained comb-growth depressing substances(16). Studies in pregnant cattle have demonstrated a preparturient increase in fecal excretion of androgens(17).

These reports led us to suspect that fecal androgen might be related to progesterone metabolism. This paper describes experiments which indicate that fecal androgen in the bovine is dependent on exogenous and/or endogenous progesterone metabolism.

Methods. Progesterone was administered to a 6-months-old Holstein calf, a yearling Jersey heifer, a Jersey cow in 7th month of pregnancy, 3 non-pregnant Jersey cows, and an 18-months-old Holstein bull. Feces were collected before, during, and after progesterone administration, dried in oven at 45°C, and pulverized in a ball-mill. The dried feces from each collection period was then well mixed and 1250 g portions taken for assay. Total weight of feces excreted from day to day did not vary appreciably in a given animal. The androgen content of each fecal sample was determined by measuring comb response of White Plymouth Rock cockerels to the fecal sample incorporated as 10% of control ration. Fecal samples were substituted for an equivalent amount of alfalfa meal present in the control ration(17). Comb response was expressed as 100 times the ratio of comb weight in mg to body weight in g. For comparison, response of chick comb to androstane-3, 17-dione and to Δ4-androstene-3,17-dione at the level of 10 mg/kg of feed is presented. Recently, androgens present in bovine feces have been identified as androstane-3, 17-dione, Δ 4-androstene-3, 17-dione, and Δ 1, 4-androstadiene-3, 17-dione(18). One g of progesterone in olive oil was given subcutaneously, daily, for 3 successive days to each animal with exception of the pregnant cow which received one g progesterone daily for 5 successive days. Control samples of feces were collected for a 2-day period preceding injections. Fecal samples were then collected during and after progesterone administration on the following days:

	Days
Holstein calf	0–1, 2–4, 5–7, 8–10
Yearling heifer	0–2, 3–5, 6–8
Jersey cow # 544	1, 2, 3, 4, 5, 6
" " # 310	1, 2, 4, 5, 6, 7, 8
" " #10160	1, 2, 3, 4, 5, 6
Holstein bull	1, 2, 3, 4, 5, 6, 7
Pregnant cow	1, 5

Results. Feces of untreated 6-months-old calf exhibited a slight but insignificant androgenic response when fed to baby chicks; whereas marked, significant androgenic responses were obtained from feces of the yearling heifer, 2 of the non-pregnant Jerseys and the pregnant Jersey cow. These data indicate that androgen elaboration in the cow is dependent upon sexual maturation or pregnancy. Feces from the untreated pregnant cow contained the greatest amount of androgenic activity. The peak of fecal androgen excretion thus synchronizes with the period of maximum progesterone production by the animal. It is interesting to note that an insignificant amount of androgen is excreted in feces of the untreated bull as previously reported(19).

Administration of progesterone to the 6-months-old calf, the yearling heifer, the 3 non-pregnant Jersey cows, and the Holstein bull produced statistically significant increases in androgenic content of feces. Upon progesterone withdrawal, the androgen content of feces gradually diminished to or below the pre-treatment levels.

Progesterone administration to the pregnant cow produced a slight but not statistically significant augmentation of androgenic content of feces. A diminution in fecal androgenic activity on prolonged administration of progesterone was indicated in the fecal sample collected after 5 daily doses of progesterone. This slight initial increase as well as the ensuing decrease in androgenic activity on continued progesterone injection might be due to a depression of endogenous progesterone

TABLE I. Excretion of Fecal Androgen in Normal and Progesterone-Treated Bovine.

		Bovine feces	Assay chicks				
Animal	Progesterone treatment	Collections (days)	No. of cockerels	Avg body wt (g)	Avg comb wt (mg)	Comb ratio ± S.E.	% diff. after inj.
Holstein calf (6 mo old)	Before inj.	2	17	271.2	200.8	74.1 ± 9.7	—
	After " *	0– 1	19	313.4	327.7	102.8 ± 10.1	+ 39
		2– 4	18	299.4	306.9	99.6 ± 12.0	+ 35
		5– 7	15	292.9	223.0	75.2 ± 7.8	+ 1
		8–10	14	284.6	156.4	54.6 ± 4.7	– 26
	Control chicks		17	279.6	158.0	55.9 ± 6.7	
Jersey heifer (yearling)	Before inj.	2	20	311.9	355.5	114.0 ± 8.1	—
	After " *	0– 2	20	328.2	539.8	162.8 ± 11.2	+ 43
		3– 5	20	308.0	444.2	144.2 ± 11.0	+ 27
		6– 8	18	324.4	384.8	120.0 ± 12.7	+ 5
Jersey cow (pregnant)	Before "	2	15	268.9	401.6	150.6 ± 17.9	—
	After " †	0– 1	16	240.3	413.0	168.2 ± 15.8	+ 12
		4– 5	18	263.7	284.2	107.9 ± 9.9	– 28
	Control chicks		14	283.9	104.9	36.6 ± 3.5	
	Androgen-fed chicks‡		19	257.7	169.7	65.5 ± 5.6	
Jersey cow #310	Before inj.	2	21	276.1	166.6	59 ± 6.3	—
	After " *	1	24	284.5	130.6	46.3 ± 3.4	– 22
		2	22	290.6	160.4	55.4 ± 4.0	– 7
		4‖	24	296.6	264.5	89.3 ± 8.4	+ 51
		5	24	284.0	158.9	55.4 ± 4.6	– 7
		6	24	309.1	153.7	50 ± 5.4	– 15
		7	25	308.6	141.7	46 ± 3.1	– 22
		8	22	295.8	130.6	44 ± 3.6	– 25
	Control chicks		23	298.7	158.2	52 ± 4.4	
	Androgen-fed chicks§		23	293.7	442.8	150.8 ± 12.2	
Holstein bull	Before inj.	2	22	327.0	200.9	61.8 ± 5.1	—
	After " *	1	21	320.9	182.7	58 ± 5.6	– 6
		2	22	337.0	246.9	74 ± 5.6	+ 20
		3	23	358.0	271	75.6 ± 6.0	+ 23
		4	22	349.1	534.4	154 ± 11.4	+149
		5	21	324.2	267.9	82.6 ± 9.9	+ 34
		6	24	353.7	199.8	56.6 ± 4.2	– 8
		7	25	334.2	155.8	47 ± 4.1	– 24
	Control chicks		20	308.6	162.3	52.8 ± 5.2	
	Androgen-fed chicks§		23	345.6	524.4	149.1 ± 13.2	
Jersey cow #10160	Before inj.	2	25	248.7	192.0	76.6 ± 5.4	—
	After " *	1	19	267.6	235.5	87 ± 5.0	+ 13
		2	24	261.8	189.2	71.9 ± 4.4	– 6.5
		3	24	256.4	175.5	67.0 ± 3.8	13
		4	23	274.5	292.4	100 ± 10.1	+ 30
		5	24	269.9	297.9	109.7 ± 7.9	+ 42
		6	23	279.4	309.8	110 ± 8.5	+ 43
	Control chicks		24	276.2	116.1	41.3 ± 2.0	
	Androgen-fed chicks§		23	271	403	143 ± 14.5	
Jersey cow #544	Before inj.	2	21	256.5	250	96.9 ± 5.9	—
	After " *	1	25	264.5	205	75.8 ± 6.7	22
		2	21	289.5	254.4	88.9 ± 7.0	8
		3	24	269.5	288.4	106.4 ± 7.9	+ 9
		4	22	263.0	280	105.5 ± 8.4	+ 9
		5	22	278	358	127.1 ± 10.8	+ 31
		6	20	273.5	318.8	112.0 ± 13.4	+ 15
	Control chicks		24	252	148.1	58.4 ± 4.7	
	Androgen-fed chicks§		21	274	410	151.3 ± 16.9	

* 1 g progesterone admin. daily for 3 successive days. † 1 g progesterone admin. daily for 5 successive days. ‡ Chicks fed androstane-3,17-dione, 10 mg/kg feed. § Chicks fed Δ^4-androstene-3,17-dione, 10 mg/kg feed. ‖ Third day fecal sample not collected.

TABLE II. Effect of Oral Administration of Progesterone on Male Chick's Comb.

Conc. progesterone in feed (mg/kg)	No. of cockerels	Avg body wt (g)	Avg comb wt (mg)	Comb ratio ± S.E.
0	23	298.7	158.2	52 ±4.4
80	22	312.3	140.6	44.8±4.9
160	25	306.8	114.1	37 ±2.4

secretion which would normally be at a maximum in late pregnancy (Table I).

Discussion. These data demonstrate a statistically significant increase in excretion of fecal androgen in the male and non-pregnant female bovine following injection of progesterone. It is suggested that the androgen found in feces of sexually active bovine female is a metabolite of progesterone and is significantly increased upon injection of exogenous progesterone.

Since androgen is bioassayed by feeding feces to chicks, it has been suggested that progesterone might be excreted as such in the bovine and then converted to an androgen in the chicks' gut, liver or other organ. However, oral administration of progesterone to chicks at a level of 40 mg/kg of feed did not stimulate comb growth(16). This report was verified by the authors at progesterone levels of 80 and 120 mg/kg of feed (Table II).

In general, two possible modes of biogenetic conversion of progesterone to androgens in the bovine may be outlined: (1) Conversion of progesterone by microorganisms of the bovine gastrointestinal tract(2), the 17-hydroxylation of progesterone (adrenal gland?) followed by oxidative degradation of the C_{17} side chain. Further research is now underway to elucidate these or other possibilities.

Summary. Sexually mature and pregnant cows excrete in their feces appreciable amounts of androgen, whereas males and immature females do not. To determine whether progesterone might be the precursor of the androgen, 7 male and female bovines were given subcutaneously 1 g progesterone daily for 3 or more days. Following these injections a statistically significant increase in fecal androgen was observed in 6 animals while a non-significant increase was observed in the pregnant cow. These data are believed to indicate that at least a part of the fecal androgen normally found in the sexually active bovine may be derived from progesterone.

The authors are indebted to Chas. Pfizer & Co., for generous supplies of progesterone and androstanedione-3,17.

1. Venning, E. H., and Browne, J. S. L., *Endocrinology,* 1937, v21, 711.

2. Buxton, C. L., and Westphal, U., PROC. SOC. EXP. BIOL. AND MED., 1938, v41, 284.

3. Hamblen, E. C., Cuyler, W. K., and Hirst, D. V., *Endocrinology,* 1940, v27, 169.

4. Dorfman, R. I., Ross, E., and Shipley, R. A., *ibid.,* 1948, v42, 77.

5. Ungar, F., Dorfman, R. I., Stecher, R. M., and Vignos, P. J., *ibid.,* 1951, v49, 440.

6. Venning, E. H., and Ripstein, *Proc. Canad. Physiol. Soc.,* 1947, 43.

7. Sommerville, I. F., and Marrian, G. F., *Biochem. J.,* 1950, v46, 285.

8. Boscott, R. J., *Ciba Foundation Colloquia on Endocrinology,* v2, 327.

9. Hill, D. L., Peterson, W. E., and Cohn, S. H., *J Dairy Sci.,* 1954, v37, 355.

10. Rogers, J., and McLellan, F., *J. Clin. Endocrinol.,* 1951, v11, 246.

11. Pearlman, W. H., *J. Biol. Chem.,* 1946, v166, 473.

12. Pearlman, W. H., and Cerceo, E., *ibid.,* 1948, v176, 847.

13. Grady, H. J., Elliott, W. H., Doisy, E. A., Jr., Blocklage, B. C., and Doisy, E. A., *ibid.,* 1952, v195, 755.

14. Miller, W. R., and Turner, C. W., *J. Dairy Sci.,* 1953, v36, 296.

15. ———, 1954, unpublished data.

16. Gassner, F. X., *Recent Progress in Hormone Research,* Academic Press Inc., N. Y., 1952, v7, 165.

17. Turner, C. W., *J. Dairy Sci.,* 1948, v31, 1032.

18. Miller, W. R., Turner, C. W., Fukushima, D. K., and Salamon, I. I., The Identification of C_{19} Steroids in Bovine Feces, *J. Biol. Chem.,* in press.

19. Turner, C. W., *J. Dairy Sci.,* 1947, v30, 1.

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Lack of Synergistic Relationship Between Thyroid and Salivary Gland Function. (21965)

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Kirkwood *et al.*(1-3) have postulated a direct relationship between activity of thyroid gland and ability of salivary glands to clear iodine. This group also believes that salivary glands play an important role in deiodination of such intermediates as diiodotyrosine (DIT) and in the control of the circulating blood level of thyroid hormone. They have demonstrated a monoiodotyrosine iodinase system in rat salivary glands, and concluded that the reverse of this reaction might actually be taking place in the intact glands. To test this hypothesis, Fawcett and Kirkwood(3) followed the deiodination of DIT in salivariectomized and intact rats. and found that DIT was not deiodinated to any appreciable extent in the salivariectomized animals.

Our preliminary observations(4) support the conclusion reached by Freinkel and Ingbar(5,6) that the iodide clearance function of salivary glands is probably not influenced directly by the thyroid, even though the amount of iodide cleared by these glands appears to correlate with the activity of the thyroid. The following data are offered as additional evidence for this conclusion, and for the belief that the deiodination of such thyroid analogues as diiodotyrosine does not take place in the salivary glands.

Methods. Carrier-free doses of I^{131} labeled diiodotyrosine, triiodothyronine, thyroxine and iodide were administered intravenously 15 μc/kg body weight to mongrel dogs maintained under sodium pentobarbital anesthesia. Saliva samples were collected under Mecholyl stimulation, and chromatographed for 18 hr on paper, together with 20% trichloracetic acid filtrates of plasma in both 80% phenol and in 68:2:30 butanol-acetic acid-water mixture. In the rat experiments, 50 μc doses of I^{131} diiodotyrosine (DIT) were injected into jugular veins of rats anesthesized with sodium pentobarbital. Blood samples were withdrawn by cardiac puncture and by tail-vein sampling one hour after the DIT injection. 20% trichloracetic acid filtrates were prepared from these specimens, and chromatographed for 18 hr with the same solvents used in dog experiments. After chromatographs were dry, each strip was cut into 10 mm horizontal sections, and these were counted in a Nancy Wood Scintillation Well Counter to an accuracy of $\pm$ 5%. Activities for each spot were then totalled and used for subsequent calculations of the % deiodination taking place and of iodide clearance.

Results. Irrespective of which of the 4 substances was administered, only I^{131} iodide was

TABLE I. Constancy of Salivary Clearance of I^{131} Iodide Irrespective of whether Iodide or DIT I^{131} Was Administered, and whether Exogenous Thyroid Hormone Was Fed in the Diet.

	"clearance"					
	Iodide injected		DIT injected		Iodide injected	
Time	Dog A	Dog B	Dog C	Dog D	Dog E before thyroid feeding	Dog E after thyroid feeding
15 min.	—	—	33	40	17	19
2 hr	26	20	34	31	19	26
4 "	27	19	20	40	—	—
8 "	22	19	20	25	14	16
12 "	24	20	24	33	—	—
24 "	27	17	—	—	16	21

* Clearance is expressed as cc of plasma cleared of I^{131} iodide per min. and is given by the expression: $\text{Clearance} = \frac{(\text{cpm/cc saliva})(\text{cc saliva/min.})}{(\text{cpm/cc plasma})}$.

TABLE II. Rate of DIT Deiodination Determined from Comparison of I^-/DIT Counts on Plasma Paper Chromatograms.

Time	% I^{131} activity appearing as iodide in plasma
30 min.	30
1 hr	59
2 "	70
4 "	75
6 "	75
8 "	80
12 "	78
24 "	80

found on the saliva chromatograms, indicating that thyroid analogues are not cleared by the salivary glands in detectable amounts. By comparing the I^{131} iodide activity found on plasma and saliva chromatograms, clearance into the saliva remained relatively constant over a 24 hr period as shown in Table I. Furthermore, feeding of thyroid substance at 0.5% of diet for 2 months, failed to influence the clearance of I^{131} iodide in the dog (Table I). Therefore the amount of iodide cleared by salivary glands appears to be a function of the plasma level of iodide, and increased blood levels of thyroid hormone do not alter this clearance.

Kirkwood *et al.* have implied that salivary glands are the principal site of deiodination. We repeated this experiment in salivariectomized and intact dogs. It was found that salivariectomized animals deiodinate DIT at the same rate, and to the same extent as control animals subjected to mock surgery. This deiodination takes place very rapidly, but 10-20% of the circulating activity 24 hr later is still DIT (Table II).

Slices of some tissues and organs of the dog were then incubated in plasma with carrier-free DIT at 37°C. At the end of 3 hour incubation period, trichloracetic acid filtrates of the media were chromatographed on paper, and the relative amounts of iodide and DIT I^{131} determined. Of all tissues analyzed, only the liver and kidneys possessed significant deiodinase activity (Table III). On a weight basis, liver appeared to be more active than kidney, and considering the relatively greater size of this organ, it might be assumed that the liver is the principal deiodination site. To determine this experimentally, however, the deiodination of DIT was followed in plasmas of nephrectomized dogs. It was found that deiodination takes place just as rapidly and completely in the nephrectomized animal as in the control. Additional evidence for the relatively minor role played by the kidney is given by the fact that appreciable amounts of DIT can be found in the urine of intact animals for several hours after the administration of DIT.

TABLE III. Relative Distribution of DIT Deiodinase in Tissues of Dog.

Tissue	% I^{131} activity as iodide	Wt of tissue slice (mg)
Liver	17.6	346
Kidney	6.5	404
Spleen	2.3	320
Submaxillary gland	2.5	294
Parotid "	2.4	356
Sublingual "	2.4	289
Skeletal muscle	2.7*	270

* Approximately 2% of Abbott Laboratory DIT preparation is in the form of iodide as determined by our chromatographic methods.

Because the data obtained in our dog studies conflict with those of Kirkwood *et al.* for the rat, we attempted to repeat their rat experiments, using the same experimental technics which they described(3). In the first experiments however, we employed cardiac rather than tail-vein blood samples. We also prepared trichloracetic acid filtrates of plasma and chromatographed these together with the whole plasma samples on the same paper, using the butanol-acetic acid-water mixture employed by Kirkwood. Deiodination took place as rapidly and extensively in salivariectomized rats as in animals subjected to mock surgery. In subsequent experiments, tail-vein samples were compared with cardiac samples taken from salivariectomized and control animals, and it was observed that essentially all the activity present in the tail-vein samples could be attributed to DIT. Our explanation for this discrepancy is that under sodium pentobarbital anesthesia, the animals become acutely cyanotic and the blood pressure falls markedly. Therefore, tail-vein samples are not representative of the systemic circulation, and any DIT passing into the tail might become trapped and never recycled to the liver for deiodination. Slices and homogenates of

the principal tissues of the rat (excluding the thyroid) were also assayed for deiodinase activity. As in the case of the dog, only the liver and kidneys were found to possess activity.

Summary. It is believed that the salivary glands play no active role in metabolism of such thyroid analogues as DIT in the dog. The amount of iodide cleared into the saliva appears to be strictly a function of the circulating plasma level of iodide ion and is not influenced by exogenous thyroid hormone. This clearance will depend of course upon the rates with which exogenous diiodotyrosine, thyroxine and triiodothyronine become deiodinated. Since neither dog nor rat salivary glands appear to contain a DIT deiodinase system such as that found in liver and kidney tissue, it is concluded that these glands are not involved in the metabolism of DIT nor in the control of the circulating blood level of this analogue.

1. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, 1954, v209, 249.

2. Thode, Harry G., Jaimet, Charles H., and Kirkwood, Samuel, *New Eng. J. Med.*, 1954, v251, 129.

3. Fawcett, D. M., and Kirkwood, S., *Science*, 1954, v120, 547.

4. Ruegamer, W. R., *Fed. Proc.*, 1955, v14, 274.

5. Freinkel, N., and Ingbar, S. H., *J. Clin. Invest.*, 1953, v32, 1077.

6. ———, *New Eng. J. Med.*, 1955, v252, 125.

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Incorporation in Adrenal Cortex of C^{14} Labeled Fractions of *Klebsiella pneumoniae*.*† (21966)

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Crude polysaccharides from *Klebsiella pneumoniae* type B produce severe joint and mild cardiac valvular lesions in the guinea pig(1). To investigate the pathogenesis of the lesions, C^{14} labeled polysaccharides have been prepared and the distribution of the label followed in the animal tissues. The present report deals with (a) the adrenal cortical localization of the polysaccharide fractions; (b) comparison of incorporation of the polysaccharide with the whole organism and certain crude protein fractions in the adrenal; and (c) attempts to establish the mechanism.

Methods and materials. *Klebsiella pneumoniae* type B organisms were grown for 16 hours on peptone-glucose agar plates containing either 1-C^{14} sodium acetate or uniformly labeled d-glucose in concentrations ranging from 2 to 25 μc per ml of media. Bacteria were gently scraped from the agar. Crude polysaccharides were obtained by acid (A) and alkaline (B) extraction technics of Wong (3). The debris (C) remaining after acid extraction was separated into 2 fractions by cold trichloracetic acid to a concentration of 5% (w/v); the supernatant (D) was dialyzed against running water and evaporated to dryness over a steam bath. A portion of the trichloracetic acid precipitate (E) was washed with cold trichloracetic acid, then extracted 2 times with trichloracetic acid at 90°C for 10 minutes as for isolating nucleic acid (F)(4). Fractions (E) and (G) were insoluble in water and 0.85% sodium chloride and have not been studied in animals. The maximum absorption of the nucleic acid fraction (F) was at 260 mμ. Except for (D), the fractions were dissolved in 1% concentration in 0.85% sodium chloride, autoclaved and injected in less than 1 ml total volume into the ear veins of male and female guinea pigs

* A portion of the data herein presented was given at the meeting of the Fed. Am. Soc. for Exp. Biol.(2), San Francisco, April 1955.

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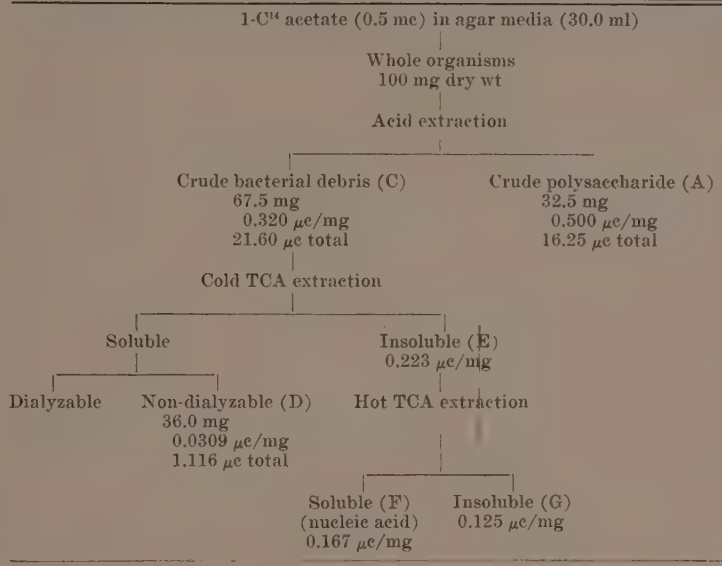
TABLE I. Incorporation of C^{14} into Certain Fractions of *Klebsiella pneumoniae*.



weighing 200-250 g at the beginning of the experiment. Whole bacteria were suspended in 0.85% saline, autoclaved and similarly injected. The lethal toxicity of the whole killed bacteria, and the bacterial debris (C), prevented the injection of quantities greater than 1.0 and 0.3 mg respectively. Urine was collected for the first 48 hours and at various daily intervals thereafter. During the collection of the urine, the guinea pigs were placed in glass containers and fed only cabbage supplement instead of their usual stock diet (Purina rabbit pellets). Animals were weighed weekly and sacrificed at varying intervals up to 2 months. Duplicate samples of organs or tissues were taken; one set was used for histologic examination and radioautography, the other set for grinding with distilled water in a Potter homogenizer, plating and counting in the proportional region with a windowless flow counter. The per cent incorporation in tissues or organs is expressed in two ways: (a) per total organ or tissue; (b) per g of dry tissue per kg body weight, ((activity per g tissue/total dose) x (body weight) x 100). The latter affords a unit comparison of the activity in different organs and tissues, is useful for tissues of uncertain total weight such as lymph nodes, and compensates for the gain in organ weights with growth. Radioautographs were made by sandwich technics on no-screen x-ray emulsions with exposures up to 3 months at 4-6°C. This provides fair histologic detail, but unsatisfactory cytologic detail. For comparison with guinea pigs, 6 mice were injected with acid-extracted polysaccharide (A) by tail vein and sacrificed at 1, 3, 7, 14, 30, and 60 days and examined as above. As controls for the labeling of adrenal tissue with noncolloidal material, 1-C^{14} sodium acetate, d-glucose, d-sucrose, and dl-sodium glutamate 1-C^{14} were heat-sterilized and given alone and with 2 mg of labeled acid-extracted polysaccharide (A) in comparable isotopic amounts. To determine the chemical association of the incorporated crude polysaccharide in the adrenal cortex, the protein and lipid fractions of adrenal homogenates were separated by trichloracetic acid to 4% concentration (w/v) and ethyl ether extraction respectively. The radioactivity of the various fractions from these 2 procedures was determined.

As a control for reticuloendothelial activity, colloidal Au^{198} was injected into 3 animals sacrificed at 1, 3, and 7 days; tissue activities were determined and radioautographs were

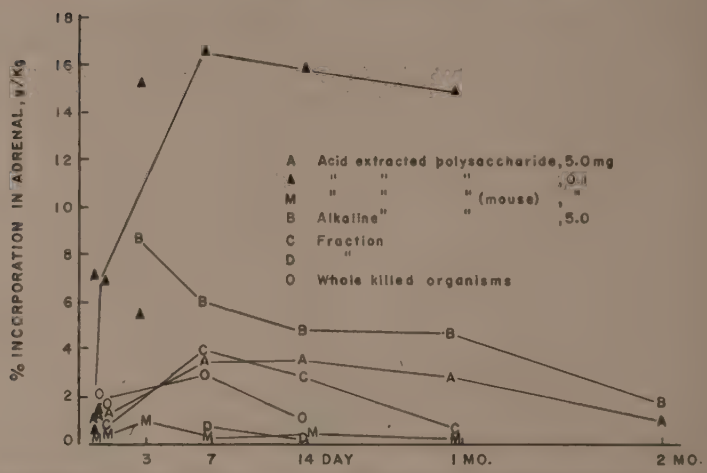


FIG. 1. Comparison of incorporation of various fractions of *Klebsiella pneumoniae* into the adrenal.

made in the same manner as for the other animals. Autoclaved undiluted Higgins india ink in ½ ml quantity was injected into 3 guinea pigs sacrificed at 3, 7, and 14 days; routine microsections were prepared.

Results. Incorporation into fractions of bacteria: The C^{14} of the acetate in the media is incorporated into all of the fractions of the bacteria which have been studied. The crude acid-extracted polysaccharide (A) has the highest activity and the cold TCA-soluble fraction (D), the lowest (Table I). The crude polysaccharide obtained by alkaline-extraction (B) has activity of 0.183 μc/mg in comparison with the crude bacterial debris activity of 0.3955 μc/mg. Extraction procedures have not been carried out on this bacterial residue. The polysaccharides obtained by the acid and alkaline extraction technics have a chemical composition similar to that previously reported(1).

Incorporation into organs and tissues of animals: After intravenous injection in guinea pigs of the various bacterial fractions, little remains in the plasma after 7 days (Table II). There is no appreciable labeling of the erythrocytes or leukocytes. The greatest loss in the urine occurs with the crude bacterial debris (C) and the nucleic acid (F). About the same percentage loss occurs with large as with small doses of the acid-extraction polysaccharide (A) (Table II). The activity in the urine parallels the activity in the plasma and is not in the form of carbonate or bicarbonate. The organs having the greatest activity, whether on total organ or unit-weight basis, are the adrenal, spleen, liver, lung, and cervical lymph nodes. The latter are consistently higher than the tracheobronchial or mesenteric lymph nodes; the mesenteric node is listed in Table II, for comparison with other studies. After the injection of the 2 crude polysaccharides, (A) and (B), the activity in the adrenal is well maintained for 4 weeks and is present in considerable concentration by the end of 2 months. By contrast, the activity in the spleen, liver, lung, and other organs declines after the first week and is barely detectable at the end of one month and not detectable at 2 months. While the greatest quantity is usually in the liver, the adrenal has by far the highest concentration per gram of tissue. The radioautographs reveal this activity to be in the zona fasciculata of the cortex and to be absent in the medulla (Fig. 2). In animals dying during the first day, the adrenals are engorged and microsections occasionally disclose zones of hemorrhage and minute areas of necrosis, usually in the inner cortex. In animals sacrificed at subsequent intervals, no histologic changes are detectable even though the radioactivity is relatively

TABLE II. % Incorporation in Guinea Pig Tissues at Optimal Time, 1 and 2 Weeks.

	I.V. dose, mg	Weeks	Plasma Total	Plasma g/kg	Adrenal Total	Adrenal g/kg	Spleen Total	Spleen g/kg	Liver Total	Liver g/kg	Lung Total	Lung g/kg	Mesenteric lymph node, g/kg	Pituitary, g/kg	Loss in urine, %
Acid extraction polysaccharide (A)	5.0	1	.14	.08	1.0	5.0	2.6	6.6	27.6	2.4	.6	.4	1.7		12
		2	.10	.05	1.2	6.1	1.4	3.5	11.9	1.0	1.1	.8	.3	.3	14
	.1	1	.09	.05	3.6	17.9	.8	2.0	18.9	1.6	.2	.1	.9	.7	7
		2	.00	.00	4.5	22.2	1.2	3.1	8.0	.7	.0	.0	.0		14
Whole, killed bacteria	.5	1	.11	.06	.8	3.9	.6	1.7	10.9	.9	.4	.3	.1	2.2	6
	1.0	2	.04	.02	.2	1.2	.4	1.1	6.0	.5	.1	.1	.1	.0	8
Alkaline extraction polysaccharide (B)	5.0	1	.17	.09	1.8	8.6	.4	1.1	2.5	2.1	.5	.3	.1	.0	14
		2	.03	.02	1.4	6.6	.3	.7	2.8	2.4	.2	.2	.2	.0	17
Bacterial debris (C)	.3	1	.25	.13	.8	4.1	.5	1.2	13.6	1.2	.2	.1	.1	.1	26
		2	.16	.09	.5	2.6	.3	.7	8.1	.7	.0	.0	.1	.0	73
Nucleic acid (F)	1.0	1	.03	.14	.0	.1	.0	.0	.0	.0	2.0	.1	.0	.0	31
		2	.00	.00	.0	.4	.0	.0	.0	.0	.4	.3	.0	.0	27
TCA soluble (D)	12.0	1	.07	.05	.1	.7	.4	1.0	4.9	.4	.1	.1	.1	.0	16
		2	.00	.00	.1	.6	.2	.4	3.5	.3	.0	.0	.2	.0	24

high. The simple extraction procedures with water, fat solvents, and trichloracetic acid show there is no significant labeling of lipid, but do not establish whether the labeled substances are protein or carbohydrate or both. Further investigation is, of course, needed to determine the nature of the labeled moieties. There is no appreciable adrenal incorporation of C^{14} from glutamic acid, glucose, sucrose, or acetate. In the spleen, the activity is in the red pulp, but not the Malpighian bodies and in the lymph nodes the radioactivity is present in the reticuloendothelial areas and not the lymph follicles. The distribution in mice is not the same as in guinea pigs, there being a greater activity in a gram of liver than of the adrenal or spleen.

India ink and colloidal gold: Variable sized particles of carbon from the india ink are found in the lung, liver, and spleen in the expected area. Some thromboses of pulmonary arteries and capillaries contain considerable black pigment. Fine black particles occur in the hepatic cord cells. Only traces of pigment can be detected in the microsections of adrenal. This pigment is in the sinusoidal epithelium and rarely in the cortical cells. The total activity and amount of colloidal gold given to the guinea pigs is not recorded, but the ratios of organ activities per gram dry weight at one week are as follows: Adrenal 1.0, spleen 23.36, liver 14.2, lung 0.13, mesenteric node 0.087, and pituitary 0.0157. Thus, colloidal gold does not have the distribution of the bacterial fractions and differs from the distribution of india ink particles in the lung and adrenal.

Discussion. The distribution of C^{14} labeled polysaccharides in the guinea pig is similar to that observed in mice and rabbits with I^{131} and dye-tagged antigen(5-7) and with fluorescent antibody(8) with the exception of a remarkably high concentration in the adrenal cortex of the guinea pig. In current theory there are three mechanisms for incorporation of the C^{14} label into the adrenal cortex: (a) cellular phagocytosis and storage of certain large molecular moieties of the nonhomogeneous bacterial fractions, (b) incorporation of a simple molecular constituent into a protein or carbohydrate component of

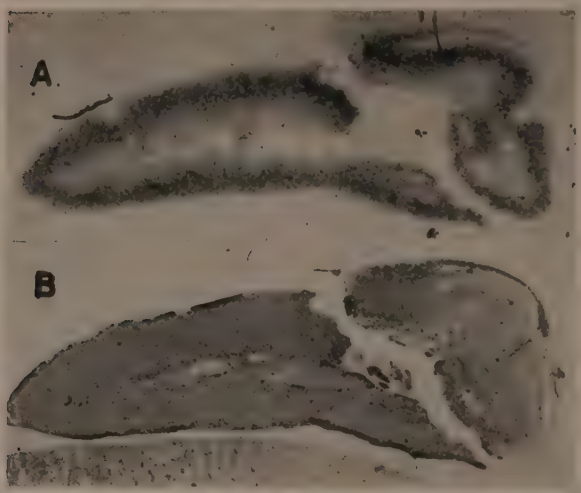


FIG. 2. Radioautograph of adrenal: (A.) of guinea pig given 5.0 mg of acid extracted polysaccharide. Comparison with H & E stained microsection. (B.) reveals activity to be in outer cortex with slight activity in inner cortex and no activity in medulla. Exposure 6 wk on no-screen x-ray film.

the adrenal cortical cells, and (c) metabolic utilization of some simple molecular constituents such as a hexuronic acid.

The long persistence of the radioactivity in the adrenal argues against a metabolic utilization of a simple molecular constituent. The radioactivity also persists in the adrenal while disappearing from the accepted sites of more active reticuloendothelial activity such as spleen and liver. Studies on the behavior of colloidal substances do not clarify the appearance of the label in the adrenal cortex. Size alone has some bearing upon the localization in the different tissues of the reticuloendothelial system(9,10), particles of larger size showing rapid deposition in the liver and spleen and colloids of smaller particle size being deposited more slowly but in greater concentration in the bone marrow and spleen. In the former study(9), the adrenals contained only 0.002% of injected dose as compared to the bone marrow value of 44.0; spleen, 1.3; and liver 3.7%. Further study of the nature and behavior of colloids is needed to explain the differences in localization and storage of protein and polysaccharide antigens and of the various colloids of greater particle size. The values in mice as well as guinea pigs are quite different from the observations of antigen localization of Friedlander's capsular polysaccharide in mice(8) and of I^{131} protein antigens in rabbits(7).

The present study shows a rough parallelism between the degree of labeling of the bacterial fraction and the concentration of C^{14} in the adrenal. For example, the acid-extraction polysaccharide (A) comprises 32.5% by dry weight and 42.9% of the C^{14} in the bacteria, while the TCA-soluble fraction (B) of debris represents 36% by weight and 0.29% of the radioactivity. Depending on dosage given, the optimal polysaccharide label incorporation (% g/kg) in the adrenal is 6 to 22 while that of fraction (D) is but 0.7 (Table II). As yet a similar parallelism between reducing sugar content of the bacterial fraction and the concentration in the adrenal has not been established, but some chemical affinity of the adrenal cortex, whether for colloids or smaller molecules, is apparent.

The incorporation of such foreign bacterial products may prove to be of considerable significance in so-called stress responses, in the electrolyte disturbance and carbohydrate metabolism with infections, antigen antibody relationships, "collagen-vascular" diseases, and in ascorbic acid metabolism.

Summary. In the guinea pig the adrenal

cortex is the site of the greatest concentration of C^{14} after the intravenous injection of labeled polysaccharide and protein fractions of *Klebsiella pneumoniae*. This concentration persists for two months, while the activity of liver, spleen, lung and other organs declines and disappears. In general, the bacterial fraction with the highest incorporation of C^{14} from the media yields the greatest incorporation in the adrenal.

1. Jones, R. S., Carter, Yolande, and Rankin, J. deW., *Brit. J. Exp. Path.*, 1954, v35, 519.

2. Jones, R. S., and Carter, Yolande, *Fed. Proc.*, 1955, v14, 407.

3. Wong, S. C., PROC. SOC. EXP. BIOL. AND MED, 1938, v38, 107 & 110.

4. Gale, E. F., and Folkes, J. P., *Biochem. J.*, 1953, v53, 483.

5. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmage, D. W., *Fed. Proc.*, 1951, v10, 553.

6. McMaster, P. D., and Kruse, H., *ibid.*, 1951, v10, 564.

7. Latta, H., Gittlin, D., and Janeway, C. A., *Arch. Path.*, 1951, v51, 260.

8. Hill, A. G. S., Deane, H. W., and Coons, A. H., *J. Exp. Med.*, 1950, v92, 35.

9. Dobson, E. L., Gofman, J. W., Jones, H. B., Kelley, L. S., and Walker, L. A., *J. Lab. & Clin. Med.*, 1949, v34, 305.

10. Zilversmit, D. B., Boyd, G. A., and Brucer, M., *ibid.*, 1952, v40, 225.

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Thioctic Acid Content and Pyruvate Oxidation in Ethionine-Damaged Rat Livers.* (21967)

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Recent investigations have established the role of alpha-thioctic acid (lipoic acid) (6,8 dithio-octanoic acid) as essential cofactor for metabolism of alpha-keto acids in both bacteria and animal tissue. This vitamin was first described by Kidder and Dewey as "Protogen," an essential growth factor for *Tetrahymena geleii*, present in liver and yeast; more recently its identity with "Pyruvate Oxidation Factor" of O'Kane and Gunsalus and "Acetate Replacing Factor" of Guirard has been established. The compound has been crystallized from liver and chemically synthesized. Thioctic acid is essential for oxidation of pyruvate by cells of *Streptococcus fecalis*, grown on thioctic acid-free medium, and is intimately associated with pyruvic oxidase and alpha-keto glutaric oxidase recovered from pigeon breast and pig heart muscle respectively. The role of this compound has been defined in the transfer to coenzyme A of the 2-carbon acetyl fragment obtained by oxidative decarboxylation of pyruvate and the 4-carbon succinyl fragment resulting from oxidative decarboxylation of alpha-keto glutarate. These developments have recently been extensively reviewed by Reed(15) and Gunsalus(7). The role of thioctic acid in animal nutrition has not been defined. No symptoms can be induced, nor can growth be diminished, in rats, by feeding a diet deficient in thioctic acid and simultaneously depressing possible intestinal bacterial synthesis of thioctic acid by feeding antibiotics(21). Feeding of an antimetabolite, the 8-methyl analogue of thioctic acid, which inhibits growth for some bacteria, produces no symptoms in chicks and rats(22). Impaired alpha-keto acid metabolism in severe human hepatic dysfunction (1, 2,19) and in experimental hepatic necrosis in rats(12,16) has been reported, and the importance of sulfur-containing amino acids in maintaining integrity of the liver is well recognized(5,16).

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Therefore it was decided to investigate thioctic acid content of liver and its relationship to pyruvate metabolism in hepatic injury produced in rats by prolonged feeding of ethionine(9). This amino acid is the ethyl analogue of methionine and interferes with utilization of methionine in protein synthesis(18).

Method. Female rats of the Wistar strain, weighing 110 to 150 g, were placed on following diet, to which dl-ethionine was added to a final concentration of 0.5%: Sucrose 75%, vitamin-free casein (Nutritional Biochemical Corp.) 16%, salt mixture (HMW) 4%, and corn oil 5%. Thiamine 10 mg, niacinamide 40 mg, pyridoxine 10 mg, riboflavin 20 mg, calcium pantothenate 40 mg, choline chloride 300 mg, menadione 5 mg, Vit. A 60000 units, and irradiated ergosterol 8500 units were added to each kg of diet. Other rats were simultaneously maintained on above diet without ethionine and were either fed *ad libitum* or pair-fed in amounts equal to those consumed by ethionine-fed animals. After 21 to 35 days on the diet, and after a 16- to 20-hour fast, the rats were killed by decapitation. At sacrifice, blood was collected from the neck and pyruvic acid determined by the method of Friedemann and Haugen(4). Livers were immediately excised and homogenized in 9 parts of chilled isotonic sucrose in glass homogenizer at 4°C. The homogenate was centrifuged 10 minutes at 600 x g to sediment nuclei and cellular debris. Supernate was then centrifuged at 9000 x g for 10 minutes to precipitate the mitochondria. The mitochondrial preparation was resuspended in 2 ml of isotonic sucrose per original gram of wet liver and reprecipitated at 14000 x g for 10 minutes. Mitochondria were then resuspended in isotonic KCl. Both kidneys were also excised and homogenized in 15 ml distilled water. Mitochondrial pyruvate oxidation was measured by the Warburg method, with flask contents as in Table I. Mitochondrial protein was determined, in quadruplicate, by the method of Lowry *et al.* (11). Thioctic acid determination was accomplished, according to the method of Stokstad *et al.*(20), by turbidometric assay of the stimulation of growth of *Corynebacterium bovis.*‡ The original medium was modified to stimulate maximal growth by addition of 1.5 g of enzyme-hydrolyzed casein/liter of double strength medium. The enzyme hydrolyzed casein (Trypticase-Baltimore Biological Laboratories) and acid hydrolyzed casein (Vitamin-free Casamino Acids-Difco) were adsorbed with charcoal, extracted with benzene at pH 3, and then readsorbed with charcoal at pH 6 to remove any residual thioctic acid. Dl-methionine, 0.5 g, was added to each liter of double strength medium to counteract any inhibition of growth of test organism which might be produced by ethionine, as previously described for *Escherichia coli*(8). However, no significant inhibition of growth of *Corynebacterium bovis* due to ethionine, at concentrations ranging from 10^{-3} to 10^{-5} M, could be detected. To free biologically-inactive, bound thioctic acid(6,13), 1 ml aliquots of liver or kidney homogenate and 1 ml aliquots of mitochondrial suspension containing 5 to 10 mg of protein/ml were autoclaved with 1 ml of 12 N H_2SO_4 for 3 hours at 120°C and 15 lbs pressure. The digests were neutralized with concentrated KOH and diluted to final concentration of 0.4 to 4.0 mγ of d-alpha-thioctic acid/ml. Urines, collected from fasting rats kept in metabolism cages for 48 hours, were diluted to a similar concentration. The diluted samples were filtered and 2.5 ml aliquots were added to 2.5 ml of double strength medium, in quadruplicate, so that final concen-

TABLE I. Hepatic Mitochondrial Pyruvate Oxidation.*

Diet	No. of animals	Mean Qo_2/mg protein ± S.E.
Control, fed *ad lib.*	3	66 ± 5.8
" , pair fed	7	69 ± 2.7
Ethionine	13	36 ± 1.3

* Results expressed as μl O_2 taken up/hr/mg mitochondrial protein (based on oxygen uptake in first 10 min.).

Flask contents: Na pyruvate .02 M; Na fumarate 6.7 × 10^{-4} M; KCl .05 M; Mg SO_4 .002 M; ATP .001 M; DPN 2 × 10^{-4} M; Niacinamide .01 M; Cytochrome C 1 × 10^{-5} M; PO_4 .02 M, pH 7.35. Mitochondrial suspension containing 8-12 mg protein.

Flask vol.: 3 ml, 20% KOH in center well.

Temperature, 37°; gas phase, O_2; thermequilibration, 5 min.

‡ We are indebted to Dr. E. L. R. Stokstad of the Lederle Division, Amer. Cyanamid Co., for the stock culture of *Corynebacterium bovis* and generous supplies of dl-α-thioctic acid.

TABLE II. Hepatic Mitochondrial Oxidation.*

Diet	No. of animals	Substrate: Pyruvate 60 μM Fumarate 2 μM	Substrate: Fumarate 2 μM	O_2 uptake due to added pyruvate
Control, pair fed	5	69 ± 2.8	32 ± 2.4	37 ± 2.7
Ethionine	6	37 ± 2.2	28 ± 3.0	9 ± 2.7

* Results expressed as mean μl O_2 uptake/hr/mg mitochondrial protein ± S.E. Flask contents as per Table I, with pyruvate omitted as indicated.

tration of d-alpha-thioctic acid ranged from 0.2 to 2.0 mγ/ml. The tubes were then inoculated with one drop of a saline-washed 48-hour culture of *C. bovis,* grown in basal medium enriched with thioctic acid. Inoculated tubes were incubated 88 hours at 30°C, and growth measured turbidimetrically in a photospectrometer. Optical density, prior to inoculation, of the relatively dark solutions obtained by liver digestion, was subtracted from the optical density following incubation, to find the increment in turbidity due to bacterial growth. A standard curve, obtained by measuring growth resulting from addition of known amounts of synthetic dl-alpha-thioctic acid‡ (having one-half the biologic activity of naturally occurring d-isomer) to the basal medium, was established simultaneously for each group of samples. At final concentrations of 0.2 to 2.0 mγ d-alpha-thioctic acid/ml, values obtained from unknowns were reproducible without a constant tendency to upward or downward "drift"; values falling outside above range were discarded.

Results. Rats fed the diet containing ethionine lost weight more rapidly than pair-fed controls. At autopsy, the livers were grossly enlarged, pale, firm, and granular. Microscopic examination revealed lobular disarrangement, marked variation in cell size, cellular swelling and cytoplasmic coagulation, with marked inflammatory cell and fibroblastic infiltration and slight fibrosis, as previously described by Koch-Weser and Popper(9). Kidneys were enlarged, soft, and pale. Microscopic examination revealed marked tubular hydropic degeneration. A similar lesion occurring following acute injection of ethionine has been described(23).

Impaired pyruvate metabolism in intact animals, following prolonged ethionine feeding, was suggested by a significant ($p<.02$) terminal elevation in fasting blood pyruvate levels from 2.9 ± .39 mg% in pair-fed controls to 4.2 ± .29 mg% in ethionine-fed animals. Pyruvate oxidation by mitochondrial preparations from livers of animals fed ethionine was markedly diminished (Table I). While mitochondria from livers of control animals maintained uniform pyruvate oxidation rate for over 40 minutes, the rate of pyruvate oxidation by mitochondria obtained from livers of ethionine-fed animals declined progressively during each 10 minutes of incubation. Hourly oxygen uptake per mg of mitochondrial protein was calculated on the basis of oxygen consumption during the first 10 minutes, to minimize above difference. The endogenous oxygen consumption by mitochondria in absence of substrate was negligible. When pyruvate was omitted from the flasks, the sole remaining substrate was 2 μmoles of fumarate "sparker" per flask. When oxygen uptake in presence of fumarate alone was subtracted from oxygen uptake in presence of fumarate plus pyruvate, the added oxygen consumption due to presence of pyruvate was markedly greater in mitochondria from livers of control animals than in ethionine-fed rats (Table II). These differences were highly significant ($p<.001$).

The thioctic acid content of liver per gram wet weight was essentially similar for rats fed chow *ad libitum,* and for rats fed ethionine-free diet *ad libitum* and in limited amounts (Table III). The mean value for control animals of 1.2 γ of d-alpha-thioctic acid per g wet liver is of the same order of magnitude as previously reported values(6,13). However, a marked decrease in thioctic acid content to 0.4 γ/g was found in livers of ethionine-fed rats. When the observed thioctic acid content was corrected for the increase in liver size noted in ethionine-fed rats, by expressing the

TABLE III. Thioctic Acid Content* of Rat Organs.

Diet	Liver			Kidney		
	No. of animals	γ/g wet wt	γ/100 g initial body wt	No. of animals	γ/g wet wt	γ/100 g initial body wt
Chow	7	1.2 ± .10	—	—	—	—
Control, fed *ad lib.*	13	1.1 ± .07	4.0 ± .27	6	1.1 ± .12	1.2 ± .11
" , pair fed	10	1.2 ± .12	4.6 ± .38	9	1.4 ± .10	1.3 ± .10
Ethionine	15	.4 ± .05†	1.7 ± .21	9	1.3 ± .11	1.3 ± .10

* Results expressed as γ of d-α-thioctic acid/g of wet organ and as γ of total liver or kidney. d-α-thioctic acid/100 g of rat body wt prior to beginning experimental diet. (Mean ± S.E.)
† For difference between livers of pair fed controls and ethionine-fed animals, $p<.001$.

results in terms of total liver d-alpha-thioctic acid/100 g original body weight, prior to starting on diet, the presence of an absolute decrease in hepatic thioctic acid from 4.6 to 1.7 γ of d-alpha-thioctic acid per 100 g of original body weight was established, at a highly significant ($p<.001$) level (Table III).

Thioctic acid content of mitochondria obtained from livers of ethionine-fed animals was also significantly ($p<.001$) diminished, from 27 to 8 mγ of d-alpha-thioctic acid/mg of mitochondrial protein (Table IV). Hepatic depletion of thioctic acid was apparently uniform in the mitochondria and in other portions of the cell, since the percentage of total hepatic thioctic acid which could be recovered from mitochondria was approximately equal in both control and experimental animals. The values of 67 and 62% respectively coincide closely with the 64% reported by Reed and Cormier(14).

Kidney was found to contain approximately 1.3 γ of d-alpha-thioctic acid/g wet tissue; this value approximately equals the amount found in liver and confirms the observations of Reed, *et al.*(13) who found approximately equal amounts of "Acetate Replacing Factor" activity in rat liver and kidney. No significant relative or absolute diminution of renal thioctic acid content could be detected in animals fed ethionine (Table III), in spite of anatomic damage to the kidneys. This is compatible with previous reports that the chemical dysfunction induced by ethionine intoxication is not as severe in kidney as in liver, the incorporation of amino acids into proteins following ethionine injection being much more impaired in liver than in kidney(18).

The 24-hour urine excretion of thioctic acid was slightly greater in fasting ethionine-fed rats than in pair-fed controls. The values of 330 and 230 mγ of d-alpha-thioctic acid per 24 hours respectively fall within the normal range of 100 to 500 mγ per 24 hours previously observed by Stokstad, *et al.*(21). The difference between the 2 groups, however, is significant only at the .05 level. Apparently, a general impairment of thioctic acid synthesis does not exist in ethionine-fed rats.

Diminished pyruvate oxidation by liver mitochondria from ethionine-fed rats could not be restored by *in vitro* addition of 1 γ of dl-alpha-thioctic acid to the flask mixture. Injection of 60γ of dl-alpha-thioctic acid into ethionine-fed rats at 96, 72, and 48 hours prior to sacrifice did not increase hepatic mitochondrial thioctic acid content above that found in untreated animals (Table IV).

Discussion. The demonstrated defect in mitochondrial pyruvate oxidation is probably not due to thioctic acid depletion. It is known that thiamine pyrophosphate, diphosphopyridine nucleotide, thioctic acid, coenzyme A, and specific protein apooxidases are all required to maintain pyruvate oxidation(15). DPN has been observed to diminish in livers of ethionine-fed rats(17). Coenzyme A originates in part from methionine and cystine (24) and is diminished in livers of rats fed a diet deficient in sulfur-containing amino acids (3, 12). Since ethionine interferes with normal metabolism of methionine, it is possible that the coenzyme A content of mitochondria is also diminished. Moreover, ethionine diminishes protein synthesis(18) and is incorporated in synthesized abnormal proteins (10); therefore the apoenzymes required for pyruvate oxidation may be deficient. In spite of the observed depletion of mitochondrial

TABLE IV. Thioctic Acid Content* of Liver Mitochondria.

Diet	No thioctic acid injection			Inj.† with thioctic acid	
	No. of animals	Mitochondrial thioctic acid content	% of total liver thioctic acid recovered from mitochondria	No. of animals	Mitochondrial thioctic acid content
Control, fed *ad lib.*	5	32 ± 2.7	—	—	—
" , pair fed	10	27 ± 2.6	67 ± 8	3	25 ± 3.0
Ethionine	10	8 ± 1.0	62 ± 5	3	6 ± 1.5

* Results expressed as mγ d-α-thioctic acid/mg mitochondrial protein (mean ± S.E.).
† Injected with 60 γ dl-α-thioctic acid 96, 72, and 48 hr prior to sacrifice.

thioctic acid in these animals, addition of thioctic acid *in vitro* does not increase the diminished pyruvate oxidation observed in liver mitochondria. Probably, a variety of factors other than thioctic acid depletion probably contribute to the pyruvate oxidation defect.

A general impairment of thioctic acid synthesis does not exist in ethionine-fed rats and therefore cannot account for diminished hepatic thioctic acid content. Since thioctic acid injection does not increase thioctic acid content of liver mitochondria in ethionine-fed rats, it appears possible that thioctic acid binding mechanism of liver may be damaged by ethionine feeding, possibly due to impaired synthesis of apoenzymes to which thioctic acid is bound, or due to impaired synthesis of enzymes which catalyze protein binding of thioctic acid or its conversion to the form in which it is biologically active.

Summary. Terminal blood pyruvic acid level was elevated in rats with liver lesions induced by feeding a diet containing ethionine. Pyruvate oxidation by mitochondria from livers of these rats diminished. The hepatic content of thioctic acid was decreased relative to control animals; the thioctic acid content of liver mitochondria was also reduced. *In vitro* addition of thioctic acid did not stimulate the diminished hepatic mitochondrial pyruvic acid oxidation. Undiminished urinary excretion of thioctic acid, failure of thioctic acid injection to increase the diminished hepatic mitochondrial thioctic acid content, and normal kidney thioctic acid content suggest that total body thioctic acid synthesis is unimpaired but that hepatic thioctic acid "binding" is impaired, in rats fed a diet containing ethionine.

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1. Amatuzio, D. S., and Nesbitt, S., *J. Clin. Invest.*, 1950, v29, 1486.

2. Carfagno, S. C., De Horatius, R. F., Thompson, C. M., and Schwarz, H. P., *New Eng. J. Med*, 1953, v249, 303.

3. Chernick, S. S., Moe, J. G., and Schwarz, K., *Fed. Proc.*, 1955, v14, 191.

4. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, v147, 415.

5. Goettsch, M., *Ann. N. Y. Acad. Sci.*, 1954, v57, 839.

6. Gunsalus, I. C., Struglia, L., and O'Kane, D. J., *J. Biol. Chem.*, 1952, v194, 859.

7. Gunsalus, I. C., *J. Cell. Comp. Physiol.*, 1953, v41, supp. 1, 113.

8. Harris, J. S., and Kohn, H., *J. Pharm. and Exp. Therap.*, 1941, v73, 383.

9. Koch-Weser, D., and Popper, H., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 34.

10. Levine, M., and Tarver, H., *J. Biol. Chem.*, 1951, v192, 835.

11. Lowry, O. H., Rosebrough, N. J., Farr, L. A., and Randall, R. J., *ibid.*, 1951, v193, 265.

12. Olson, R. E. and Dinning, J. S., *Ann. N. Y. Acad. Sci.*, 1954, v57, 889.

13. Reed, L. J., DeBusk, B. F., Johnston, P. M., and Getzendaner, M. E., *J. Biol. Chem.*, 1951, v192, 851.

14. Reed, L. J., and Cormier, M. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 724.

15. Reed, L. J., *Physiol. Rev.*, 1953, v33, 544.

16. Schwarz, K., *Ann. N. Y. Acad. Sci.*, 1954, v57, 878.

17. Shank, R. E., Mendeloff, A. I., and Karl, M., *J. Clin. Invest.*, 1953, v32, 603.

18. Simpson, M. V., Farber, E., and Tarver, H., *J. Biol. Chem.*, 1950, v182, 81.

19. Smith, L. H., Ettinger, R. H., and Seligson, D., *J. Clin. Invest.*, 1953, v32, 273.

20. Stokstad, E. L. R., Hoffman, C. E., and Belt,

M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 571.
21. Stokstad, E. L. R., Broquist, H. P., and Patterson, E. L., *Fed. Proc.*, 1953, v12, 430.
22. Stokstad, E. L. R., *ibid.*, 1954, v13, 712.
23. Wachstein, M., and Meisel, E., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 648.
24. Yang, C. S., and Olson, R. E., *Fed. Proc.*, 1954, v13, 482.

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Destruction of Virus Hemagglutination Inhibitor of Egg-White by Newcastle Disease Virus.* (21968)

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The viruses of mumps, influenza and Newcastle disease cause agglutination of certain mammalian red blood cells (RBC) by connecting these cells and forming a lattice-like matrix. If the ratio of virus and RBC is controlled, the reaction can be observed visually. In most instances the virus elutes or dissociates from the RBC after an indefinite time following mixing of the virus and red cells. The virus-treated cells are no longer capable of being "rebridged" by the virus but the virus retains its capacity to act again on untreated cells(1).

It is now known that normal ferret serum, serum from other animal species(2), a variety of tissue suspensions(3), ovalbumin(4), cow's milk(5), etc., will inhibit viral hemagglutination (HA). Inhibitor present in all of these substances seems to be very similar in composition to the "virus receptor" substance of the surface of erythrocytes which is commonly regarded as mucoprotein(6,7). Viruses of the mumps-Newcastle-influenza group can enzymatically destroy the inhibitor common to these materials. The exact role of this enzymatic action in infection has not been determined. One hypothesis is that firm adsorption to the cell surface is needed to allow the virus to enter the cell.

Since so many variations in characterized properties, *e.g.*, pathogenicity, heat stability of the hemagglutinin, of strains of Newcastle disease virus (NDV) exist, a study of the enzymatic activity of several strains of this virus seemed warranted. The following studies have utilized crude egg-white as the substrate or HA inhibitor in determining the mucinase activity of NDV.

Materials and methods. Viral strains. A repository for strains of NDV, maintained in the Department of Veterinary Science, University of Wisconsin, with the cooperation of the Agricultural Research Service, U. S. Department of Agriculture and the Newcastle Disease Technical Committee of the North Central States Region, has more than 80 strains, including 1 from Australia, 8 from Canada, 2 from Mexico, 1 from Japan, 8 from Europe and the remainder from the United States. All repository strains and the N strain of Dinter(8) were tested for their ability to destroy egg-white inhibitor (EWI). For the rate studies, however, only 7 strains were employed: Vic-Australia-1932, B1, Hert-Eng-1933, Milano-Italy-1945, GB-Texas-1948, (H)Najarian-Ohio-1948 and Roakin-NJ-1946. The Lee strain of influenza B virus was used as the indicator virus in the hemagglutination-inhibition (HI) tests. White Leghorn chick-

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en eggs from a commercial hatchery were used. As far as could be determined, these eggs came from flocks not vaccinated or infected with Newcastle disease.

Viral pools. Newcastle disease and N viruses were prepared by inoculating 10 or 11-day-old chicken embryos, intraallantoically, with 0.05 ml of a 10^{-2} dilution of stock, allantoic-fluid virus. Inoculated eggs were incubated at 98 to 99°F and candled 2 to 3 times daily. From embryos that had died after 24 hours, allantoic fluids were harvested and pooled. If the fluids were to be used within 4 to 5 days, they were stored at 4°C; otherwise at –20°C. The Lee strain of influenza B virus was prepared by inoculating 10 to 11 day-old, embryonated eggs, intraallantoically, with 0.05 ml of a 10^{-2} dilution of stock, allantoic-fluid virus. After further incubation at 98 to 99°F for 65 hours, the living embryos were chilled overnight and allantoic fluids harvested and handled as described for the other viruses. Calcium chloride ($CaCl_2$) 1 g, sodium chloride (NaCl) 9 g, boric acid (H_3BO_3) 1.203 g, and sodium tetraborate ($Na_2B_4O_7 \cdot H_2O$) 0.052 g were dissolved in 1 liter of distilled water, pH 7.0 ± 0.2. *Hemagglutination tests.* Starting with a 1 to 5 dilution of allantoic-fluid virus, serial 2-fold dilutions were made in 0.25 ml volumes of physiological saline contained in 13 x 75 mm tubes. Chicken RBC, 0.25 ml of a 1% suspension in physiological saline, were thoroughly mixed with each dilution. The test was read after 30 to 45 minutes at room temperature. The endpoint, 1 HA unit, was the highest dilution of virus showing 1+ agglutination. One plus HA was not evident in most instances since the last tube to show agglutination was usually 4+. Consequently, most 1+ values were obtained by interpolation, *e.g.*, if the highest dilution of virus showing agglutination was the 1 to 320 dilution and the degree of agglutination was 4+ the 1+ value was 320 plus three-fourths the difference between 320 and the next dilution, 640. The result is a value of 1 to 560 for 1+ agglutination.

Hemagglutination-inhibition test. Starting with a 1 to 100 dilution of EWI solution, serial 2-fold dilutions were made in 0.25 ml volumes of physiological saline contained in 13 x 75 mm tubes. Five HA units of indicator virus contained in 0.25 ml were mixed with each dilution. After 30 minutes at room temperature, 0.25 ml of a 1.0% suspension of chicken RBC was added to each tube and the HA patterns ascertained 45 minutes later. The endpoint was considered as the last tube to show complete inhibition of agglutination. *Egg-white inhibitor or ovomucin.* The "thick" albumin of fresh, White Leghorn eggs served as the source. Stock ovomucin was a 5% solution in calcium borate, buffered saline. Inhibitory activity was determined by HI testing using 5 HA units of heated Lee virus as the indicator antigen. One HI unit was considered as the smallest quantity of inhibitor required to completely inhibit the activity of 1 HA unit of the heated, Lee strain of virus. *Indicator virus.* Allantoic fluid from eggs infected with the Lee strain of influenza B virus was heated at 56°C for 30 minutes. This heated virus has the capacity to combine with inhibitor but not the ability to destroy it. Consequently, heated Lee virus serves well as a sensitive indicator for inhibitor titrations.

Results. In exploratory experiments employing 7 strains of NDV, each was effective in inactivating EWI substance. Subsequently, the entire collection of NDV strains in the repository and the N strain of Dinter(8) were tested for their ability to destroy EWI. Tests were conducted by adding 20 HA units of each virus to 1 ml of a 1:100 dilution of raw egg-white and incubating the mixture at 37°C for 15 hours. The reaction was terminated and virus activity eliminated by heating the mixture at 65°C for 30 minutes. The content of each tube was assayed for EWI content by an HI test employing heated, Lee strain of influenza B virus as the indicator. With each strain of NDV 20 HA units completely inactivated the inhibitor contained in 1 ml of 1:100 dilution of egg-white.

A series of experiments was designed to study certain kinetic features of the reaction between EWI and NDV. Strains Vic, Hert-E, Milano, GB, Roakin, Najarian and B1 were selected for the first experiment in which the rate of inactivation of 1280 HI units of EWI by each strain was determined as a function of its HA concentration. The reaction was car-

ried out at 37°C for 90 minutes after which time the reaction was stopped by heating as indicated. The content of each tube was assayed for residual EWI by HI testing with heated, Lee B indicator virus. Under the conditions of the experiments, the inactivation rates differed among strains and was dependent on the virus concentration within strains. For the Hert-E, Milano and B1 strains, only 32 HA units were required to completely destroy the inhibitor whereas 64 units of the Najarian and GB strains and 128 units of the Vic and Roakin strains were required.

The next experiment was to determine, as a function of time, the rate of inactivation of 1280 HI units of EWI by various quantities of 6 strains of NDV. The strains employed were the same ones used in previous experiments with the exception of the Najarian strain. Three quantities of each virus suspension were used, 256, 128 and 64 HA units. The virus and EWI mixtures were incubated at 37°C, and at 5 minute intervals for 30 minutes, a sample of each mixture was removed and heated at 65° C for 30 minutes. The residual content of EWI was assayed as in the previous experiment. The Milano, Hert-E and B1 strains not only inactivated the EWI most rapidly but behaved similarly, except for less activity with the 64 HA units concentrations with Hert-E. The inactivation rates of GB and Vic strains were similar but somewhat slower than of the Milano, Hert-E and B1. The Roakin strain did not inactivate any EWI within the 30 minute test period. In all cases where inactivation occurred, the rate of inactivation of EWI corresponded directly to the HA concentration of the virus.

The third experiment was designed to ascertain the rate of inactivation of EWI by strains of NDV as a function of EWI concentration. The strains of NDV were the same as employed in the previous experiment. Three concentrations of EWI, 1280, 640 and 320 HI units, were used and 128 HA units of each strain of virus were employed. The virus and EWI mixtures were treated exactly as described in the previous experiment. Hert-E, Milano and B1 gave similar inactivation patterns. The patterns of Vic and Roakin were similar while that of GB was intermediate between the two groups. For all strains, the rate of inactivation was fastest with the 320 HI units of EWI. The rates of inactivation with 640 and 1280 HI units approximated each other but were slower than the 320 HI unit concentration.

TABLE I. Capacity of NDV* in Allantoic Fluid Containing 0.4% Formalin to Destroy Egg-White Inhibitor.

NDV strain	Time in days after treatment with formalin					
	3	4	9	14	21	28
Vic	++†	—	—	—	—	—
Hert-E	++	++	++	++	++	±
Milano	++	++	++	++	++	—
GB	++	++	++	++	++	++
Roakin	++	++	++	—	—	—
Naj	++	++	++	++	++	++
B1	++	++	++	++	++	++
Md-II	++	++	++	++	—	—
Hert-M	++	++	++	++	++	±
Ky-50	++	++	++	++	++	++
Lederle	++	++	++	++	++	++
RO	++	++	++	++	++	—
Wagner	++	++	++	++	++	±
Appleton	++	++	++	++	++	++
CG	++	++	++	++	++	++
ViPol	++	++	++	++	++	++

* All strains of unformalinized NDV destroyed egg-white inhibitor after storage at 5°C for 25 days.

† —, ±, ++ = no, partial and complete destruction, respectively, of egg-white inhibitor in 1:100 dilution of egg-white.

The final experiment was concerned with the stability of the mucinase property of allantoic-fluid NDV when exposed to 0.4% formalin. Sixteen strains were employed. At 3, 4, 9, 14, 21 and 28 days after adding the formalin and storing the mixtures at 4°C, the ability of each strain to destroy EWI was tested. This was accomplished by adding 0.25 ml of formalinized, allantoic-fluid virus to a 1:100 dilution of ovomucin. After overnight incubation at 37°C, the mixtures were heated at 65°C for 30 minutes. The residual EWI determinations are recorded in Table I. All strains except Vic possessed mucinase activity at 4 days after formalinization and at 14 days Roakin had lost mucinase activity. The Md-II strain did not inactivate egg-white inhibitor after 21 days. After 28 days, RO was inactive, while Hert-E, Hert-M and Wagner were only partially active.

Discussion. The mucinase activity of the mumps-Newcastle-influenza group of viruses is

widely recognized. If viral mucinase has any biological significance, it remains to be defined. Many hypotheses have been proposed but none have been definitely proved. However, it is well established that viruses are intracellular parasites which must invade and enter a susceptible host cell before multiplication can occur. This immediately raises the question of the mechanism of penetration of the host cell and the relation of inhibitors to the infectious process.

Almost all cells of the body, especially those of the respiratory tract, and except those of the skin, are protected by a mucous secretion. Since the viruses mentioned above have, in the main, an affinity for respiratory tract tissues, mucinase may have as its function the destruction of this protective material in order to clear the way for the attachment of the virus to the host cell preparatory to its penetration.

Strains of NDV are known to vary markedly from one another in properties such as pathogenicity, heat stability of the hemagglutinin and neurotoxicity. For some strains these properties have been well defined. However, no one has reported any mucinase studies of the various strains of NDV with the purpose of possibly relating them to some of the known properties. This purpose prompted, in part. the studies presented in this paper.

All strains of the repository were able to destroy the hemagglutination inhibitor of hen's egg-white. After the preliminary experiment, 7 strains of NDV markedly different from each other were selected for determination of the rate of the reaction. Despite the fact that these studies were performed in a crude fashion since neither the enzyme nor the substrate were separated and purified from their native sources, the results obtained appear susceptible to logical explanation. The first concern was with the rate of inactivation of EWI as a function of virus concentration. The 7 strains employed fell into 3 groups: (1) Hert-E, Milano and B1; (2) Najarian and GB; (3) Vic and Roakin. Group 1 inactivated the inhibitor rather quickly while the other 2 were progressively less active. With the exception of the Najarian virus, these 7 strains were used in other studies of inactivation rate as follows: (1) Rate of inactivation of 1280 HI units of EWI by various quantities of NDV as a function of EWI concentration. For each study, the 6 strains fell into 3 groups which are the same groups as described for the initial rate study. The greater the quantity of virus (enzyme), the faster was the inactivation rate. For the inhibitor concentration studies, the results were slightly different since the 320 HI units were inactivated in all cases at a faster rate than the 640 and 1280 units, which were inactivated at about the same rate. Whether the slower rate for the larger concentrations of the inhibitor was due to higher viscosity of the crude substrate solution is not known. There was also observed in all of these experiments a lag period whose duration depended on the concentration of the enzyme.

The activity groups into which these strains fell appear subject to correlation with only one known property *i.e.*, the rate of their elution from red cells. There is no apparent correlation between mucinase activity and pathogenicity for chickens since 2 highly pathogenic strains, Hert-E and Milano and a non-pathogenic strain, V1 constituted the group with the greatest mucinase activity.

A study dealing with the stability of mucinase to 0.4% formalin indicated this enzymatic activity was fairly stable.

Summary. Studies were made on the presence and mucinase activity of Newcastle disease virus and of the stability of this enzyme function to formalin. (1) All strains of Newcastle disease virus studied possessed mucinase activity although the rates at which they inactivated egg-white inhibitor differed greatly. (2) The rate at which egg-white inhibitor was inactivated was dependent on enzyme (virus) concentration. The concentration of egg-white inhibitor also affected the rate of its inactivation. (3) The rate of inactivation by Newcastle disease strains of virus could be correlated only with one known property—its rate of elution from red blood cells. (4) Newcastle disease virus mucinase was fairly stable to formalin.

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1. Hirst, G. K., *J. Exp. Med.*, 1942, v76, 195.
2. ———, *ibid.*, 1942, v75, 49.
3. Friedwald, W. F., Miller, E. S., and Whatley, L. R., *ibid.*, 1947, v86, 65.
4. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 312.
5. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *ibid.*, 1949, v72, 227.
6. McCrea, J. F., *Austr. J. Exp. Biol. Med. Sci.*, 1948, v26, 355.
7. Gottschalk, A., and Lind, P., *Brit. J. Exp. Path.*, 1949, v30, 85.
8. Dinter, Z., *Tierärztliche Umschau*, 1949, p. 229.

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Importance of Optimum Amounts of Virus to Demonstrate Neutralizing Antibody Rise in Convalescent Poliomyelitis Sera.* (21969)

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(Introduced by L. Emmett Holt, Jr.)

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A study of 38 patients infected with poliomyelitis viruses showed a 5-fold or greater increase in the homologous antibody titer in 35 (92%). This finding was at variance with that of other workers(1,2), who postulated that the rise in neutralizing antibodies occurred so rapidly that often no increases in titer were demonstrable. Thus Miller and Wenner, using a virus concentration of 32 $TCID_{50}$† in their neutralization tests, observed a 10-fold rise in homotypic antibody in only 3 of 14 patients(2). Tests for homologous antibody were carried out in only 7 patients, none of whom showed a similar rise. Melnick and his associates, with a virus inoculum of 100 $TCID_{50}$, reported rises of homotypic antibody in 15 (37%) of 41 patients from whom poliomyelitis viruses had been isolated(1). Homologous antibody formation was not studied by these workers.

This report presents evidence that the demonstration of increases of homologous antibody in patients infected with poliomyelitis viruses is related to the dose of virus used in the neutralization test.

Materials and methods. Data are derived from 38 patients studied in New York City in 1953, a non-epidemic year.‡ Their stools were collected within 2 weeks of onset of illness and after concentration in the Spinco high speed centrifuge, yielded poliomyelitis viruses as follows: Type 1, 25 patients; Type 2, 2 patients; Type 3, 11 patients. All but 4 showed some degree of weakness or paralysis. Serum specimens were collected at frequent intervals and tested for neutralizing antibodies against the patient's own virus and the 3 prototypes. To be reported here are the results of tests carried out on mixtures of homologous virus and serum obtained usually in the 1st or 2nd week of illness, in the 3rd or 4th week, and lastly at about the 12th week. Trypsinized monkey kidney cells grown in tissue culture in glass tubes§ and examined microscopically for cytopathogenic changes constituted the test object for isolation of virus and for neutralization tests. Neutralization tests were carried out usually with serial 4-fold dilutions of serum, inactivated at 56°C for 30 min. Serum dilutions were mixed with an equal vol-

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† $TCID_{50}$—Tissue culture infective doses, 50% end point.

‡ We are grateful to the staffs of Willard Parker, Mt. Sinai, Babies', and Flower-5th Avenue Hospitals for supplying material for this investigation.

§ For the past year tubes containing cultures of monkey kidney cells supplied by Microbiological Associates, Inc., Bethesda, Md., have been used.

TABLE I. Relation of Increase in Neutralizing Antibody and Dose of Virus Used in Test. 38 patients.

Day of illness 1st serum obt.	Virus type in stool	Dose of virus in neut. test*	Antibody fold-increase		
7	1	3.2	500		
6	3	2.7	400		
5	3	4.0	200		
4	2	2.3	200		
2	1	2.7	200		
11	1	2.5	60		
8	3	3.7	50		
7	1	4.2	40		
11	1	3.7	40		
3	1	3.0	30		
7	1	3.5	25		
8	1	2.7	25		
18	3	3.9	25		
7	3	1.2	25		
8	1	3.7	16+?	76%	
5	3	3.2	16		
10	1	3.5	16		
7	1	4.5	12+?		92%†
7	1	1.5	12+?		
8	1	4.5	12		
8	3	2.5	12		
3	1	3.2	12		
8	1	2.0	10+?		
9	3	4.2	10		
5	3	4.0	10		
4	1	4.0	10		
6	3	3.0	10		
4	1	2.5	10		
14	2	1.0	10		
8	1	2.7	7		
9	1	4.7	7		
18	1	2.7	6	16%	
18	1	3.2	5		
9	1	2.5	5		
16	1	3.5	5		
7	1	4.0	3		
14	3	2.7	2		
10	1	4.9,3.5,2.4,1.2	0		

* Expressed as negative log. † 92% of patients.

ume of a selected dilution, usually 1:500, of the patient's virus. The latter was titrated simultaneously in each test against normal monkey serum. The mixtures were incubated at room temperature for one hour. 0.1 cc of each dilution was pipetted into tissue culture tubes containing 0.9 cc of standard medium.‖ As a rule 3 tubes per dilution were used. In some of the "box" titrations 2-fold dilutions of serum were mixed with decimal dilutions of virus and inoculated into 6 tubes per dilution. All tubes were examined daily under the microscope for cytopathogenic effects. The seventh day reading was taken as final. Antibody titers were calculated by the method of Reed and Muench and expressed as the 50% serum dilution endpoint (log of reciprocal).

Results. Table I shows the relation of degree of antibody rise and dose of virus used in the test. A 5-fold or greater increase in homologous antibody obtained in 35 (92%) of 38 patients. Twenty-nine patients (76%) had antibody rises from 10 to 500-fold. In 6 patients (16%) increases from 5 to 7-fold were obtained. It is evident that antibody increments as great as 200-fold were obtained with as much as 4 logs of virus. The average amount of virus used in all the tests was about 3 logs. Substantial increases in antibody were demonstrated even when the first serum specimen was obtained as late as the second or third week of illness. In the group whose antibody rises were under 10-fold the first serum was obtained on an average of the 12th day of illness, whereas in the group of 29 patients whose antibody rises were 10-fold or greater, the first serum was drawn on an average of 7.2 days after onset. If the reverse calculation is made, it will be seen that the average antibody rise is 95-fold in patients whose first serum was obtained in the first 7 days of illness as compared with an average of 16-fold for those whose first serum was obtained between the 8th and 18th days.

"Box" Neutralization Tests with Homologous Virus. During the beginning of this investigation in most of the individual tests a single relatively large amount of virus was used against serial dilutions of serum. It seemed possible that the greater number of definite antibody rises observed might be related to the larger doses of virus employed as compared with those used by previous observers. This possibility was investigated by simultaneous titrations of virus and serum (so-called "box" titration) from 3 patients—2 infected with Type 1 (A and B) and one with Type 3 (C). The results ap-

‖ The "standard medium" consisted of Earle's balanced salt solution, lactalbumin hydrolysate 0.5% and calf serum, 2%. 50 units of penicillin and 50 mg of streptomycin per ml of medium were added. Recently 20 units of mycostatin (Squibb) per ml were added which has helped considerably to suppress the growth of yeasts.

TABLE II. "Box" Neutralization Tests.

Patient	Day of illness serum drawn	A: Logs of *homologous* virus in test .5	.6	.7	1.5	1.6	1.7	2.5	2.6	2.7	3.5	3.6	3.7	4.5	4.6	4.7	5.6	B: Logs of *homotypic* virus in test .4	.6	1.4	1.6	2.4	2.6	3.4	3.6	4.4	4.6	5.4
		Antibody titer (log of dilution)																										
A	3	3.4+?			2.3			6, 1.2			.6			<.6														
	80	3.5			2.8			2.6, 2.4			1.7			<1.2														
B	9		3.2+?*				2.2			1.6			1.1			<.6		<.6	3.9†			2.6		2.0		1.1–?		.5–?	<.6
	82		3.6				3.4			2.8			2.8			1.1		1.0	4.0			3.2		2.5		2.1		.9	<.6
C	15			2.6+?			2.2			1.7			1.1			<.6				2.8†			2.0		1.7		1.2		.5
	93			3.2			2.8			2.2			2.2			1.1				3.2			2.7		2.4		2.2		1.5
	Fold increase		0	2 ?	?	3	16	4	100, 16	16	3	12	50	12	?	4	4	3	0	2½		4	5	3	5	10+?	10	2+?	10	0

* 6 tissue culture tubes per dilution of serum.
† *Idem*; serial 2-fold dilutions of serum.

pear in Table II (Section A). It is evident that little or no antibody rise was demonstrable with the smallest doses of virus (.5, .6, .7 log). With 1 log higher dose only 1 patient (B) showed a significant rise; with 2 logs, 2 patients, and with 3 logs, all 3 patients showed substantial rises. When the sera were "overwhelmed" with 4.5-5.6 logs of virus, no significant antibody increase resulted. In the test with patient B's virus and serum the accuracy was enhanced by using 6 tubes per dilution. In the case of patient C, a 12-fold antibody increase was the maximum achieved. This occurred with 3.7 logs of virus. It may be that this relatively small antibody rise is accounted for by the lateness of obtaining the acute phase serum, *e.g.*, 15th day of illness. In any event, the result of this test shows a trend in keeping with the pattern of the other two.

"Box" Neutralization Tests with Homotypic Virus. It was important to learn if this apparent relation between antibody rise and dose of virus would apply to ***homotypic*** as well as to homologous virus. Similar "box" titrations were performed with the sera of 2 patients, B and C, mixed with Type 1 (Mahoney) and Type 3 (Leon) viruses, respectively. To increase the accuracy of the tests, these box titrations were carried out with serial 2-fold dilutions of serum against decimal dilutions of virus and the mixtures were inoculated into 6 tissue culture tubes per dilution. The results are presented in Table II (Section B). As with homologous viruses, little or no antibody rise resulted with the smallest doses of homotypic virus. With 3.4 and 3.6 logs, 10-fold or higher increments were obtained. Unlike the other tests in which the largest doses of virus either obliterated or diminished the difference in antibody level between the two sera, in the case of patient C's sera and the heaviest concentration of Leon virus (4.6 logs), a 10-fold increase was still demonstrable. Then actual titers are not very different, however, from those obtained with C serum and homologous virus (Section A). In that test when a comparable dosage (4.7 logs) of C virus was used even though it seemed to result in a 4-fold antibody rise, the titers of each serum (0.6 and 1.1) did not differ significantly from those obtained with Leon virus (0.5 and 1.5).

From the results of these 2 tests with homotypic viruses, it would appear that the relation of antibody increase to virus dose was not limited to the patient's own virus, but a similar pattern was obtained with homotypic viruses, Mahoney and Leon, respectively.

Discussion. The results obtained in individual tests with various virus strains and sera indicated a relationship between increase in neutralizing antibody and concentration of virus used in the test. This was confirmed by "box" neutralization tests in which virus and 2 serum specimens from the same patient were titrated simultaneously. In the zones of antibody excess and virus excess, respectively, there was little or no apparent increase in antibody from acute to convalescent phase of illness. In the middle dosage range of about 3 logs of virus regular antibody increases were demonstrable. The explanation of this phenomenon is not clear. The question arises as to whether antibodies found in the acute stage differ qualitatively or quantitatively from those found in convalescence. Work is being done currently on the dynamics of virus-antibody union which may shed light on the problem. For example, is the union between virus and "late" antibody more stable than that which occurs with "early" antibody? Are there detectable differences in the velocity of the reaction? Does "late" antibody have a greater avidity for virus than "early" antibody? The results of the present report have been obtained entirely with the use of cultures of monkey kidney cells as the test object of virus activity. It is important that Steigman and Sabin(3) and Winsser and Sabin(4), who carried out quantitative neutralization tests in monkeys, observed substantial homologous antibody increases between 16 and 32-fold with amounts of virus ranging from 160 to 1000 PD_{50}. The results of the present study indicate that the relation between antibody rise and virus dose is not limited to homologous systems. The acute and convalescent sera of 2 patients (one with a Type 1 infection, the other Type 3) showed similar antibody trends against the homotypic virus, Mahoney and Leon, respectively.

Even though the mechanism by which it works is not understood at present, the phenomenon appears to have important practical as well as fundamental implications. The neutralization test may still prove to be an important diagnostic tool provided the dose of virus is properly adjusted. It would appear that approximately 1000 $TCID_{50}$ is the optimum dose of virus. Another factor of importance in demonstrating antibody increase is the time of drawing the "acute" serum sample—the earlier the better. The study of basic problems concerned with the kinetics of the poliomyelitis virus-antibody reaction will be advanced by the use of Dulbecco's new plaque method(5).

Summary. The formation of neutralizing antibodies against their own virus was studied in 38 poliomyelitis patients. In the sera of 35 (92%), 5-fold or greater antibody increases were demonstrated. Twenty-nine (76%) exhibited rises between 10 and 500-fold. The average dose of virus used in separate tests was about 3 log dilutions. "Box" neutralization tests revealed that little or no antibody increase was demonstrable in the zones of antibody or virus excess. Substantial antibody rises were obtained in the middle zones, when between 2.5 and 4.6 log dilutions of virus were used. A similar trend of antibody increase was found with homotypic virus studied in two individuals. These results seem to indicate a relation between increase in neutralizing antibody and concentration of virus in the test. The mechanism by which this phenomenon operates is not understood but it appears to have both fundamental and practical implications.

1. Melnick, J. L., Ramos-Alvarez, M., Black, F. L., Girardi, A. J., and Nagaki, D., *Yale J. Biol. and Med.*, 1954, v26, 465.

2. Miller, C. A., and Wenner, H. A., *Pediatrics*, 1954, v14, 573.

3. Steigman, A. J., and Sabin, A. B., *J. Exp. Med.*, 1949, v90, 349.

4. Winsser, J., and Sabin, A. B., *ibid.*, 1952, v96, 477.

5. Dulbecco, R., and Vogt, M., *ibid.*, 1954, v99, 167.

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Effect of Levallorphan and Nalorphine upon Barbiturate-Induced Respiratory Depression in Rats.* (21970)

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N-allylnormorphine HCl, (nalorphine), and levo-3, hydroxy N-allylmorphinan tartrate, (levallorphan), have been shown to be effective antagonists of analgetic-induced respiratory depression both clinically and experimentally(1-9). However, with respect to the possible use of nalorphine against barbiturate depression the available reports are confusing, for some authors report that nalorphine is an effective barbiturate antagonist(10-12), while others found it to be ineffective(3,13-15). No reports are available concerning the effectiveness of levallorphan as an antagonist to barbiturate depression.

This study was undertaken to determine in rats the effectiveness of both nalorphine and levallorphan as antagonists to the respiratory depressant effects of some of the more commonly used barbiturates.

Procedure. Male albino rats (Sprague-Dawley) 200-300 g in weight were used. All experiments were acute, each animal serving as its own control, and about 50 experiments were carried out. The procedure was similar to that described previously(7), and consisted of cannulation of the trachea under ether, and infiltrating the area with a local anesthetic. The animal was restrained and connected to a one-way valve system to measure tidal air. After a control period, the barbiturate under examination was given, in a dose determined previously to produce 50% respiratory depression, then either nalorphine or levallorphan were administered by intraperitoneal injection at various dosage levels. The barbiturates examined were phenobarbital, barbital, amytal, pentobarbital and secobarbital.

Results. Levallorphan exerted no demonstrable effect in reducing the barbiturate-induced depression of respiration when used in dosage ratios of 1:1 or less of levallorphan to barbiturate. Because of this ineffectiveness of levallorphan a second series of experiments was carried out in the dose of the antagonist was increased. Fig. 1. Levallorphan was effective in diminishing only the pentobarbital-induced respiratory depression, and only when twice as much of the antagonist as depressant was employed. No noticeable stimulatory effects were observed with other barbiturates.

In the experiments with nalorphine only the higher dosage levels were tested, these were identical with doses used in the second series with levallorphan. Again, as with levallorphan, nalorphine was only effective in diminishing pentobarbital-induced respiratory depression, and only when twice as much nalorphine as pentobarbital was used.

Discussion. Both levallorphan and nalorphine were found to be moderately antagonistic to pentobarbital-induced depression but only when very large doses of these 'antagonists' were employed. On the other hand, no stimulatory effects were observed when these 2 compounds were used with phenobarbital, barbital, amytal and secobarbital. This is in marked contrast to the effect of levallorphan and nalorphine upon respiratory depression induced by the narcotic analgetic compounds, where the effective ratio of antagonist to depressant ranged from 1:1 to 1:30 for Dromoran, methadone, morphine, alphaprodine and meperidine. For example, the ratios in the case of morphine sulfate depression are 1:6.7, and 1:5, and in meperidine depression 1:30 and 1:5 for levallorphan and nalorphine respectively(7).

In this present series of experiments leval-

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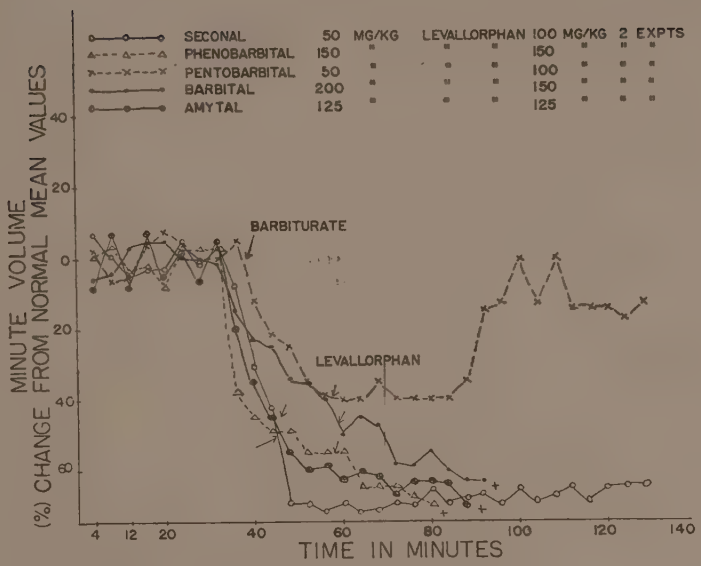

FIG. 1. Effect of levallorphan upon respiratory depression produced by various barbiturates. Effect with nalorphine was essentially the same, and the dosages were identical.

lorphan and nalorphine were not used in doses greater than 150 mg/kg because this would have exceeded the LD_{50} in rats for both compounds, (approximately 180 mg/kg for both levallorphan and nalorphine).

Eckenhoff *et al.*(13) found that nalorphine was ineffective in combating clinical barbiturate depression. However, Belford and Kao (11) and Vivante *et al.*(12) later reported that nalorphine was effective in relieving pentobarbital-induced respiratory depression in the dog. Clinically, Dulfano *et al.*(10) found nalorphine to be effective in combating barbiturate depression in several instances. On the other hand, several recent reports(3,14,15), have pointed out the failure of nalorphine, both experimentally and in man, to relieve respiratory depression from barbiturates.

There are no reports in the literature concerning the ability of levallorphan to combat respiratory depression produced by barbiturates. From the present study it would appear that both levallorphan and nalorphine can restore partially the respiratory minute volume in rats when it has been depressed by pentobarbital. However, this effect could only be achieved with very large doses.

Summary. It was observed that nalorphine and levallorphan were ineffective in antagonizing the respiratory depression produced in rats by large doses of phenobarbital, barbital, amytal and seconal. The two compounds did, in large doses, cause some diminution in the depression produced by pentobarbital.

1. Lasagna, L., *A.M.A. Arch. Int. Med.*, 1954, v94, 532.

2. Winter, C. A., Orahovats, P. D., Flataker, L., Lehman, E. G., and Lehman, J. T., *J.P.E.T.*, 1954, v111, 152.

3. Salomon, A., Marcus, P. S., Herschfus, J. A., and Segal, J. S., *Am. J. Med.*, 1954, v17, 214.

4. Benson, W. M., O'Gara, E., and Van Winkle, S., *J.P.E.T.*, 1952, v106, 373.

5. Gross, E. G., and Hamilton, W. K., *J. Lab. Clin. Med.*, 1954, v43, 938.

6. Foldes, F. F., Swederlow, M., and Siker, E. S., *J.P.E.T.*, 1955, v113, 21.

7. Costa, P. J., and Bonnycastle, D. D., *ibid.*, 1955, v113, 310.

8. Eckenhoff, J. E., Helrich, M., Hege, M. J. D., and Jones, R. E., *ibid.*, 1955, v113, 332.

9. Thomas, D. V., and Tenney, S. M., *ibid.*, 1955, v113, 250.

10. Dulfano, M. J., Mack, F. X., and Segal, M. S.,

New Eng. J. Med., 1953, v248, 931.
11. Belford, J., and Kao, F. F., *Fed. Proc.*, 1954, v13, 336.
12. Vivante, A., Kao, F. F., and Belford, J., *J.P.E.T.*, 1954, v111, 436.
13. Eckenhoff, J. E., Elder, J. D., and King, B. D., *Am. J. Med. Sc.*, 1952, v223, 191.
14. Gruber, C. M., Jr., *J.P.E.T.*, 1954, v111, 409.
15. Boyd, E. M., and Pearl, M., *C.M.A.J.*, 1955, v73, 35.

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Morphology of Characteristic Particles Associated with Avian Erythroblastosis.* (21971)

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Erythroblastosis and myeloblastosis (granuloblastosis)(1) are highly malignant leukemic diseases of viral etiology in the chicken. In the latter condition involvement of the precursors of the myelocytes(2) results in the occurrence in the blood circulation of very large numbers of primitive cells of myeloblastic features. The virus of erythroblastosis presumably affects the progenitors of the red blood cells and the disease is characterized(3) by the presence of many erythroblasts in the circulating blood. That the respective viruses, likewise, are present in the circulation in both conditions is attested by transmissibility of the diseases with filtered blood plasma. By application of ultracentrifugal procedures, the virus of myeloblastosis has been obtained from the plasma of diseased chicks in appreciable amounts in purified preparations for physical, chemical and biological characterization(4,5). The findings with myeloblastosis have stimulated the undertaking of similar studies with the virus of erythroblastosis. Examination of filtered blood plasma from chicks with this disease has revealed characteristic particles, which by virtue of the experience with myeloblastosis, may represent the etiological agent of erythroblastosis. This paper describes results of preliminary investigations on the morphology of these particles observed in electronmicrographs.

Materials and methods. Erythroblastosis virus was obtained from Dr. Astrid Fagraeus of the State Bacteriological Laboratory, Stockholm, who had received it previously from Dr. J. Engelbreth-Holm at the Department of Pathological Anatomy, University of Copenhagen. The agent has been passed in this country by means of filtered plasma injected whole, or diluted, in 0.1 ml volumes in 3-day to 3-month-old chickens. The birds employed routinely were inbred White Leghorns of line 15 developed(6) at the Regional Poultry Research Laboratory, East Lansing, Mich. and the same as those used in the work with myeloblastosis(7). As will be developed in later reports, the strain of erythroblastosis appears to be a characteristic entity entirely distinct from myeloblastosis with respect both to the pathological manifestations and to host-response to the agent. Small chicks with the disease were bled by section of the sagittal sinus and larger ones by heart puncture. The blood was received in chilled tubes containing heparin and cleared of cells by 2 cycles of low speed centrifugation. The plasma was recovered in each case with fine tipped pipettes, then filtered with celite and passed through Selas filters by the technics(8) employed in the work with myeloblastosis. Donor birds were selected on the basis of the number of erythroblasts seen in routine daily blood smears, and bleeding was effected only when the chickens were near death. Once the chicks became obviously ill, as indicated by lassitude or weakness, the condition pro-

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gressed rapidly to death within a few hours. In most instances the chickens were closely watched, and bled when they became moribund. During these stages of the disease, the increase in erythroblasts was rapid, and it seemed likely that the virus content of the plasma might, likewise, reach the highest levels in the few hours before death of the host. Thus far, none of the plasmas has exhibited significant activity to dephosphorylate adenosine triphosphate in the microscreening test (9). Consequently, this criterion of plasma content of virus, so useful in the study of myeloblastosis, has not been available in the experiments with erythroblastosis. However, in contrast with host response to the virus of myeloblastosis(10), 2-month-old chickens were not greatly different in susceptibility than 3-day-old birds to infection with erythroblastosis virus. This was a most important factor, since the older birds furnished much larger volumes of plasma as source of the virus. The ages of the donor chicks used in the present work were from 3 to 60 days. Blood was drawn 7 to 15 days after inoculation of the virus. The technics employed for examination of the particles in erythroblastosis with the electronmicroscope were identical with those applied in the studies on the virus of myeloblastosis(8). Although observations were made with the particles in plasma, the principal investigations involved the particles in concentrates obtained by 1 or 2 cycles of sedimentation. The best results were obtained with individual plasmas spun, undiluted, in collodion tubes of appropriate volume. The particles were concentrated by centrifugation at 16,400 x g for 60 minutes and resuspended in small volumes, 5 to 25% of those of the initial plasmas, in normal saline solution containing 0.001 M phosphate of pH 7.0. These concentrates from individual plasmas or, more often, in pools (pooled only after the initial concentrating procedure) were centrifuged at 7,000 x g for 5 minutes to eliminate aggregated material. In some cases the procedures of high and low speed centrifugation were repeated for a second time. Preparations were made for electronmicrography either with a drop of undiluted concentrate dried on an agar surface, or the particles were spun down on agar(8) from suspensions diluted 1-10 to 1-100. The particles were fixed on the agar surface with osmic acid vapor and examined either after shadowing with chromium or without shadowing. Examinations were made with comparable technics of the particles directly in plasma. Counts of the particles in plasma or concentrates were made by sedimentation on agar in the usual way(8).

Results. The virus of erythroblastosis is relatively highly "virulent" in the sense that the chicks of the inbred line 15 were very susceptible to infection. With the largest doses of the agent, 0.1 ml of the best plasmas, onset of the disease, marked by the appearance of polychrome erythrocytes(3), was as early as 48 hours. Death followed frequently within 4 to 5 days. This appearance of intensity of host-virus interaction occurring in the induction and progression of the disease, as compared with the process in myeloblastosis, is emphasized by the observation of relatively low concentrations of characteristic particles which might represent virus in the plasmas.

That low particle content of the plasma is characteristic of erythroblastosis has been evident from several indications. In no case has the plasma exhibited visible turbidity ascribable to the presence of particles in high concentration. Ultracentrifugation results in no visible or very small pellets. In most instances material concentrated 3 or more times with respect to the volume of the starting plasma showed little opalescence, though occasional plasmas yielded quite turbid suspensions at

TABLE I. Approximate Characteristic Particle Content of Various Individual Plasmas from Chicks with Erythroblastosis Counted Either Directly in Plasma or Estimated from Counts with Concentrates of the Agent.

Donor	Age at bleeding (days)	Interval since inoculation (days)	Erythroblasts in blood smears†	Particles/ml plasma, $\times 10^8$
O 913*	17	12	4	257
O 911*	15	10	5	6.6
N 62	17	12	3	117
O 660	17	12	4	47
N 108	19	9	8	3.3
N 225	19	9	6	27

* Estimated from counts on concentrates.
† No. of cells/oil immersion field.

FIG. 1. Electronmicrograph of particles from the plasma of chick with avian erythroblastosis obtained by sedimenting the particles on an agar surface. Particles were shadowed with chromium. Magnification 27,600 ×.

20 times concentration. Finally, approximate estimates obtained by counting the particles in concentrates, after unmeasurable loss in the sedimentation procedure, as well as more accurate determinations made by counting the particles directly in plasma showed highly variable results ranging (Table I) from about 10^8 to 10^{10} particles per ml of plasma. The data of Table I are cited as representative of the findings. Many plasmas and concentrates were examined, but counting was excluded in most instances because of aggregation or excess amorphous material extraneous to the particles in relation to the very small numbers of particles.

Despite the technical difficulties, micrographs have been obtained clearly revealing the characters of the particles. One of the best pictures is that of Fig. 1 obtained with the concentrate of plasma O 913, Table I. The particles were sedimented from 4 ml of the plasma and taken up in 1 ml of saline. After low speed centrifugation the particle content of this suspension was 1.03×10^{11} per ml. The suspension was diluted 1-100 (equivalent to a 1-25 dilution of the original plasma), and the particles were spun onto agar.

The electronmicrograph reveals a population of particles of considerable homogeneity of kind which are of variable size and, in general, of spheroidal shape. There is present, also, amorphous material obviously different from the predominant particles and relatively few particles of a different magnitude of size from that of predominant ones. Measurements on small numbers of the particles of various sizes gave the value 102 mμ as the average diameter. It is seen that the particles are not regularly rounded but exhibit surface depressions, folds and protrusions from the main body of the particles, some as short tails or flattened membrane-like structures. The general characters of the particles are clearly shown, though the preparation contained much other material.

Shadowing with metal, though increasing the contrast and revealing 3-dimensional features of virus and other particles, obscures completely all except occasional contour evidence of internal structure. Pictures of unshadowed particles are difficult to obtain, but an informative example was available in the present work. A small drop of a suspension of twice sedimented particles was placed di-

FIG. 2. Electronmicrograph of particles from the plasma of chick with avian erythroblastosis obtained by drying a drop of concentrate on an agar surface. Preparation was not shadowed. Magnification 34000 ×.

rectly on agar, dried, fixed with osmic acid and examined without shadowing. Fig. 2 shows 2 principal types of structures. One is represented by an essentially circular image with a small center of material of high electron absorbing power surrounded by a "membrane-like" substance of very low contrast. Similar images have been seen(11) with the virus of myeloblastosis, which is a particle of very low hydrated density(12) and is regarded as consisting primarily of watery, gel-like material surrounding a small region, or regions, of concentrated, relatively dense (to electrons) internal material. The images of the particles in erythroblastosis have the same appearance.

The second structures of interest are those having the appearance of empty, low contrast membranes greatly resembling particle "ghosts" consisting of the material surrounding the internal masses of the other images. In many instances there are seen empty spaces or apparent rents in the substance seeming to indicate space previously occupied by the dense material or through which it escaped. Other images have the appearance of fragments of the low-contrast material. Such images were seen not infrequently in the present studies, but the mechanism for the production of the material, that is, for the rupture of the particles and the escape of the dense constituent is not known. Although the interpretation may be faulty in detail, it seems certain that these structures of low contrast must be related to the intact particles, and do not resemble any of the usual nonspecific components of plasma either from normal birds or chicks with erythroblastosis. It is especially notable that many of the low contrast images are fairly regular in outline, are of the size of the images of the intact particles, and have the same appearance as that of the peripheral material of the specific particles.

Discussion. The present findings do not suffice to establish unequivocally the biological nature of the particles described. The component has not been observed, however, in the plasmas of normal chicks, and it occurs as a population of particles of considerable homogeneity with respect to kind only in diseased individuals. The characters of the particles and their obvious relation to the occurrence of erythroblastosis, together with considerable experience in the study of the

contents of chicken plasma in investigations of myeloblastic leukemia, constitute strong presumptive evidence that the particles represent the etiological agent of the disease.

The particles in erythroblastosis are remarkably similar to those of the virus of myeloblastosis. In the case of the latter agent, a variety of investigations has indicated that the myeloblastic leukosis virus is a spheroidal particle of low hydrated density and high water content. Most of the volume of the virus is constituted of material of low electron absorbing properties surrounding a small mass of electron-dense material, which may be regarded as nucleoprotein as in the case of analogous structures in other viruses. All of the evidence obtained in the present work is compatible with the view that the characteristic particles in erythroblastosis are essentially identical in physical structure with the agent of myeloblastosis. It would be fruitless to extend the comparison, inasmuch as sufficient amounts of the particles are accessible for analyses by other physical methods. It is desirable to note that the size value for the particles in erythroblastosis, 102 mμ, is somewhat smaller than that, 120 mμ for the agent of myeloblastosis. This difference, however, is not regarded as significant. A real difference between the two diseases is the particle content of the plasma. In erythroblastosis, the concentration of particles reaches levels of approximately 10^{10} per ml, whereas in myeloblastosis the levels(8) are as high as 2×10^{12} per ml. Another striking difference between the diseases resides in the lack of adenosine triphosphatase activity in the micro test in erythroblastosis. This does not signify a lack of enzyme activity of this virus, since the results may well have been related principally to the low concentration of the particles in the individual plasmas.

Of particular interest is the bearing of the present findings on the general problem of the etiological interrelationships among the various diseases classified(1) as members of the leukosis complex. These relationships can be determined only by intensive studies of the physical, chemical and biological properties of the agent associated with each condition. A considerable basis of information has been established with the agent of myeloblastosis(4). It would appear that the superficial physical aspects of the morphology of the particles in erythroblastosis are indistinguishable from those of the agent of myeloblastosis. Within the limits of the small number of examinations thus far made, the agent of a third form of leukosis(13), the RPL strain 12 of lymphomatosis, does not differ appreciably in morphology from the particles in erythroblastosis and myeloblastosis. This resemblance, however, may be entirely superficial, and further study may reveal significant physical and chemical differences. It would be well to emphasize the biological specificity of these 3 agents in the cell type involved; in the pathological manifestations; and in the character of host response in the induction of the respective diseases. These investigations on the agent of leukosis have been of principal value thus far in the physical demonstration of the virus entities in the plasma of birds with myeloblastosis and characteristic particles which are probably the etiological agents of erythroblastosis and lymphomatosis and of the feasibility of obtaining the particles in the state and amount needed for critical experimentation.

Summary. A particulate component, which may be the etiological virus, was isolated by ultracentrifugation of the blood plasma of chickens with erythroblastosis. The particles were of spheroidal shape and variable size, averaging approximately 102 mμ diameter. Micrographs of unshadowed preparations showed circular images of an appearance suggesting that the particles consisted predominantly of a watery, low-electron contrast, gel-like material surrounding a small relatively dense internal structure. The findings were compared with those obtained in the analogous studies of particles representing the virus of myeloblastosis and that of one form of lymphomatosis.

1. Jungherr, E., Doyle, L. P., and Johnson, E. P., *Am. J. Vet. Res.*, 1941, v2, 116.

2. Furth, J., *J. Exp. Med.*, 1931, v53, 243.

3. ———, *Arch. Path.*, 1931, v12, 1.

4. Beard, J. W., Sharp, D. G., and Eckert, E. A., *Adv. in Virus Research*, 1955, v3, in press.

5. Eckert, E. A., Sharp, D. G., Beard, D., Green,

I., and Beard, J. W., *J. Nat. Canc. Inst.*, in press.
6. Waters, N. F., *Poultry Science*, 1945, v24, 269.
7. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Canc. Inst.*, 1954, v14, 1055.
8. Mommaerts, E. B., Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *ibid.*, 1954, v14, 1011.
9. Green, I., Beard, D., Eckert, E. A., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 406.
10. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Canc. Inst.*, 1955, v15, 1195.
11. Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *J. Bact.*, 1952, v63, 151.
12. Sharp, D. G., and Beard, J. W., *Biochem. et Biophys. Acta*, 1954, v14, 12.
13. Sharp, D. G., Eckert, E. A., Burmester, B. R., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 204.

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Effects of Sodium Diphenylhydantoinate upon Isolated Small Intestine of the Rabbit. (21972)

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The effects of a number of drugs upon the rhythm of isolated rabbit intestine were studied. Among the drugs tested was sodium diphenyl-hydantoinate (Dilantin). This proved to have remarkable effects which, to our knowledge, have not been described previously.

Material and methods. The small intestine was rapidly removed from a freshly stunned rabbit. This was immediately placed into oxygenated Tyrode's solution. Lengths of duodenum were most generally employed, although jejunum and ileum were also used in some experiments. The unused portions were stored in cold Tyrode solution for later use. The length of the gut used was between 2 to 3 cm. One end of the gut was fixed by a silver hook which in turn was fastened to the walls of the chamber. The other end of the gut was led by a thread to a very sensitive myograph transducer. This in turn was connected to the amplifier of a Hoff-Geddes-Spencer Physiograph and the resultant recording was traced out by an ink-writing pen upon a moving strip of paper. The amplification factor for movement was about 200 at maximum gain. With the initial tension of the myograph spring overcome, a 200 mg weight produced a deflection of 7.5 mm at maximum gain.

The chamber enclosing the isolated gut had 3 openings at its base. The first of these allowed for the continuous entry of oxygen into the solution. This kept the solution well-oxygenated and also served to mix the solution and any added drugs. An opening at the lowest part of the chamber allowed for drainage at will and a third opening provided for the entry of fresh Tyrode solution of pH about 7.6. A further portal in the upper part of the chamber enabled one to add drugs to the solution. The bath was kept at any constant temperature between 35° and 38°C by means of a heat lamp which was shone upon a blackened portion of the chamber. The intensity of this heating was regulated by means of a variac.

Results. The addition of Dilantin in amounts sufficient to produce a resultant concentration of 2 to 8 mg% (0.07 to 0.29 mmol l) in the solution resulted in a rapid decrease in the amplitude of the contractions and a fall in tone (Fig. 1, A and B). This effect was noted within 15 to 30 seconds and the contractions generally disappeared within one minute. The addition of smaller amounts of Dilantin resulted in a more gradual decrease in the tone and in the amplitude of the contractions. A concentration of 0.5 mg % (0.018 mmol/l) was sufficient to reduce considerably the amplitude of contraction of the gut (Fig. 1, C). With the smaller concentrations of Dilantin, the gut contractions would become reduced in

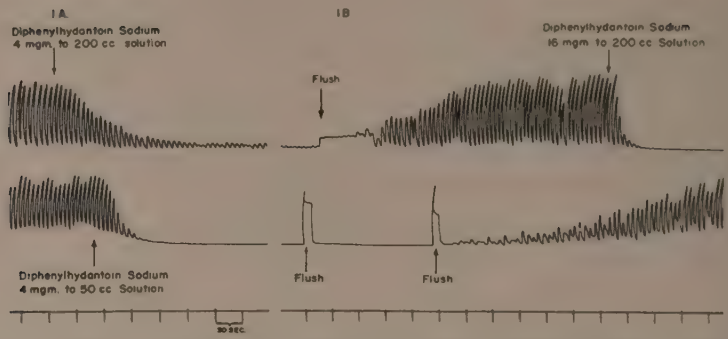

FIG. 1. A. Effects of Dilantin upon 2 separate samples of rabbit duodenum, both examined simultaneously. Note rapid decrease in amplitude of contractions and fall in tone upon addition of the drug; also note more rapid effects of higher concentrations. B. Note rapid return of contractions of normal amplitude upon flushing away the Dilantin. Gut exposed to higher concentration requires a longer time and more flushing before returning to normal level of contractions and of tone.

amplitude, but after several minutes the contractions would become fairly constant in strength.

Dilantin affects the rate as well as the amplitude of contractions. This effect is apparent in concentrations of the drug which markedly depress the amplitude of contractions without, of course, abolishing them (Fig. 2). By means of increasing the amplifying factor of the apparatus, it was possible to detect minute contractions of the gut. With concentrations of Dilantin of 6 to 8 mg%, the rate of contractions was reduced to one-third to two-thirds of the control rate.

The effects of Dilantin are rapidly reversible. Even after the gut had been exposed to concentrations of the drug sufficient to prevent any recordable contractions over a period of over 50 minutes, removing the drug solution and replacing it with fresh Tyrode solution led to the appearance of small contractions within 75 seconds and a return to normal amplitude of contractions within 3 minutes. This reversibility of effect was present after repeated exposures to the Dilantin over a period of 8 hours.

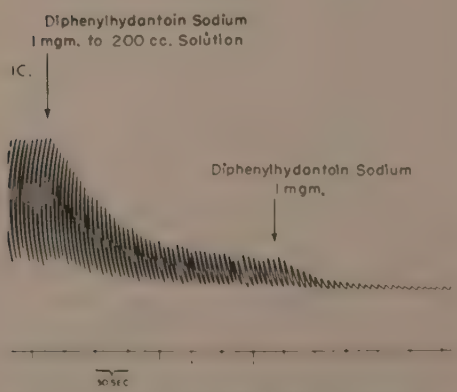

FIG. 1. C. Slower and lesser effect of smaller concentrations of Dilantin on amplitude and rate of contractions.

To rule out the possibility that any of the effects noted were due to the alkalinity of the Dilantin, sodium hydroxide solution was added to the Tyrode solution bathing the gut in amounts sufficient to equal and, in some instances, exceed the pH of a solution of Dilantin concentrated enough to stop the contractions of the gut. Such an addition of base led to an increase in tone (Fig. 3A)(1). This increase in tone could be reversed and the tone returned to normal levels by the addition of Dilantin to the already alkaline solution (Fig. 3B). This would appear effectively to rule out simple alkalinity as the basis for the mechanism of action of the Dilantin.

The effects of the Dilantin were compared with the direct effects of sodium phenobarbital upon the isolated gut. In concentrations of 10 mg% (0.39 mmol/l), the sodium phenobarbital had a slight effect in reducing the rate of contraction from 16 per minute to 14

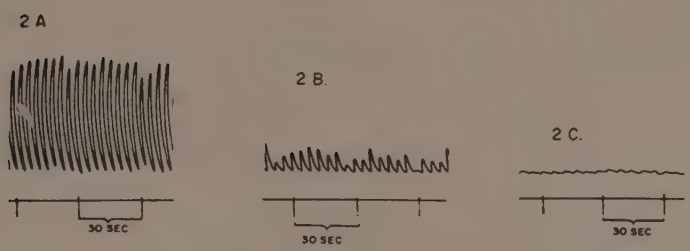

FIG. 2. A. Control record of contractions of gut. Note great amplitude of contraction and rate of 16 per min. B. In intermediate concentrations of Dilantin, amplitude of contraction is reduced. Rate is 15 per min. Amplification has been increased. C. In a concentration of 6 mg % Dilantin contractions of the gut are barely visible, even with the maximum gain of the amplifying system. The rate is 11 per min.

per minute, with some reduction in the amplitude of contraction as well. At a level of 21 mg% (0.83 mmol/l) there was a marked reduction in the amplitude of contractions and the rate was 14 per minute. The effects of the sodium phenobarbital upon the gut were much less rapidly reversible than those of Dilantin.

Discussion. A concentration of 0.5 mg% of Dilantin was effective in reducing the amplitude of contraction of the isolated small intestine of the rabbit. Concentrations of 8 mg% abolished recordable contractions.

It has been reported(2) that the blood levels of hydantoinate in human patients receiving Dilantin for the treatment of epilepsy range between 3 to 6 mg%. Thus, it is obvious that the range of concentrations em-

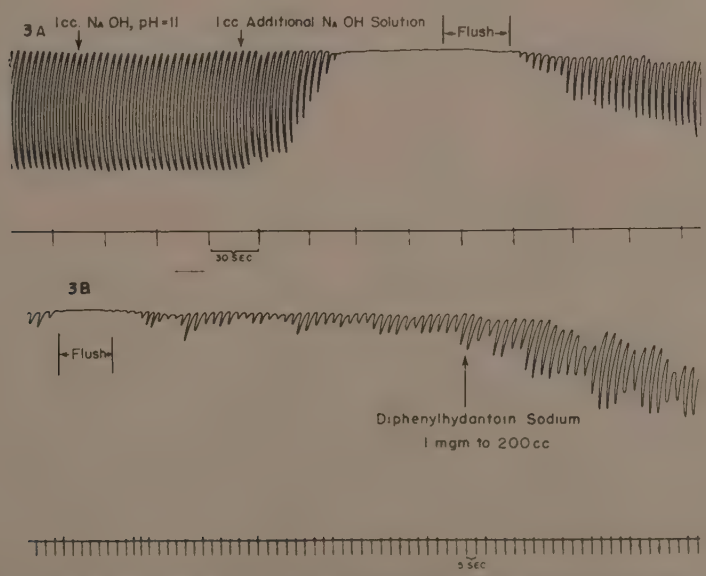

FIG. 3. A. Rise in tone upon addition of NaOH solution. Although the record suggests that contractions stopped with the increase in tone, this was not so. Gut continued contracting, but contractions were not recorded since normal tension range of this myograph was exceeded. This effect of NaOH is reversible by flushing. B. Flushing after addition of base leads to a slight reduction in tone. Addition of Dilantin leads to rapid fall in tone. Apparent increase in amplitude of contractions is artefactual and is due to tension exceeding recording range of the myograph in the earlier section of the record.

ployed in these experiments was well within therapeutic limits. This is of some practical significance, as these findings may help to explain the constipation which sometimes develops in patients taking Dilantin.

These results may also be of importance in relation to the problem of so-called "abdominal" epilepsy. Commonly accepted criteria for the diagnosis of this form of seizure include: (1) episodic attacks of abdominal pain, (2) "cerebral dysrhythmia" as revealed by electroencephalography, and (3) improvement with marked reduction or cessation of these attacks upon the institution of anti-epileptic medication, usually with Dilantin. The implication has been that improvement upon the institution of this medication is due to an effect upon the central nervous system. The possibility of such improvement being due to a direct effect of the medication upon the gut has not been given adequate consideration. If, as has been demonstrated in these experiments (*in vitro*), the Dilantin has a direct effect upon the gut (*in vivo*), then the third criterion is no longer necessarily valid in indicating the "central" origin of these attacks. The problem of "abdominal" epilepsy should be considered without regard for the effects of anti-convulsant medication, unless the direct effects of this medication upon the gut can be dissociated from its effects upon the central nervous system.

These experiments may have clinical implications in other diseases affecting the gastro-intestinal tract, particularly those where increased motility or spasticity, or both, are prominent.

Summary. (1) Dilantin has a direct effect upon isolated rabbit gut. It reduces tone, reduces the amplitude and rate of contractions, and in higher concentrations can completely abolish the contractions. (2) The effect of Dilantin upon the isolated gut is rapidly reversible by simply replacing the gut in fresh Tyrode solution. (3) This effect of Dilantin is not due to its alkalinity. (4) Dilantin has a much more marked effect upon the gut than has sodium phenobarbital. (5) The clinical implications of these effects of Dilantin upon isolated gut are discussed briefly.

1. Gruber, C. M., *J. Pharm. Exp. Therap.*, 1926, v30, 149.

2. Kozelka, F. L., and Hine, C. H., *ibid.*, 1940, v69, 292.

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Propagation of Equine Abortion Virus in the Chick Embryo.* (21973)

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Doll(1) was the first to offer unequivocal proof that the virus of equine abortion (EAV) can be cultivated in the chorio-allantoic membrane of the chick embryo with intranuclear inclusions characteristic of the disease. The hamster-adapted variant used in his experiments would not induce infection in chick embryos by allantoic, amnionic or yolk-sac inoculation. Doll has pointed out that all attempts at propagation with infected equine fetal tissue have met with failure in the chick embryo. Previous experience(2) has shown that a field strain of EAV can survive 8 serial passages in chick embryo lung and liver maintained in flasks. No inclusions could be identified in chick tissue but were demonstrated in fetal horse tissue inoculated with chick embryo passage material. In the present communication, the serial propagation of EAV in chick embryos is reported.

Material and methods. The virus used to initiate this study (strain F) had been adapted to the hamster liver(3). The liver inocula, hamster or chick embryo, were frozen and ground, diluted 1:10 in physiological saline and centrifuged lightly. Fertile eggs

* Aided by a Grant from the Grayson Foundation, Inc.

of 12-day incubation were injected with 10% liver suspension in the yolk and amniotic sacs with 0.5 ml and 0.1 ml respectively. Initially, the eggs were inoculated with hamster liver, and subsequently with chick embryo liver for 14 consecutive passages. Each serial passage was terminated 4-5 days following inoculation. In order to determine the pathogenicity of chick embryo material, alternate passages were inoculated into 3-week-old hamsters. Representative pieces of liver from the hamster and chick embryo passages were fixed in Zenker-acetic acid for histological studies.

Complement-fixation methods. The original complement-fixation procedure(4) for equine abortion employing the 50% hemolysin end-point method, was abandoned because of the excessive amount of reagents required. Doll later employed the conventional method(5). The latter procedure was used in the present study to save materials. The liver antigens were ground in physiological saline and stored at −50°C. Prior to use they were centrifuged at 2500 rpm for 10′. The origin and preparation of the positive and negative sera, and control antigens, have been previously documented(3,6). Essentially, the procedure utilized 2-fold 0.25 ml serial dilutions of antigen, 0.25 ml of 1:10 dilutions of antiserum of equine origin and 2 full units of complement in 0.5 ml. Fixation was carried out overnight at 4°C and 0.5 ml of sensitized cells were added for 1 hour at 37°C. Adequate controls were used for each reagent, including normal horse serum.

Results. Serial propagation in chick embryos. No significant mortality was observed in any of the trials. The search for intranuclear inclusions characteristic of the infection in susceptible species was unrewarding in early experiments. In the 6th and subsequent passages intranuclear inclusions were identified in liver parenchymal cells. They were indistinguishable from those seen in the horse(6). Inclusions were occasionally numerous, usually focal in distribution and not abundant.

Identification of embryo propagated virus. Hamster adapted equine abortion virus is quite pathogenic for young hamsters with LD_{50} titres varying between 10^{-6} and 10^{-8}.

TABLE I. Complement Fixation Reactions.

Antigens	Antigen titration end-points
Equine fetal lung (abortion virus)	128
Normal fetal lung	4
14th passage chick embryo liver	Anticomplementary
Normal chick embryo liver	"
Hamster liver inoculated with 14th passage chick embryo	64
Normal hamster liver	8

Intranuclear inclusions are constant and abundant in the parenchymal cells of the liver. Chick embryo passage material remained pathogenic for 3-week-old hamsters, but the results were variable. Ten per cent chick embryo liver rarely killed all inoculated animals. The liver from all dead animals contained abundant typical inclusions. Doll(7) reports that chick embryo EAV loses pathogenicity for weaned hamsters. The dilution of the original hamster material during serial passage in chick embryo exceeded 10^{-15}. The dilution is only approximate since the routes of inoculation preclude accurate calculation. The known limiting dilution of infectivity was greatly exceeded, showing that the agent has been propagated in the chick embryo. The chick embryo adapted virus was further identified by the complement-fixation reaction. The data shown in the table present proof that the specificity of the egg adapted virus was maintained. The antigen titration end-point is defined as the highest dilution of antigen which resulted in complete fixation of complement with a 1:10 dilution of antiserum of equine origin.

Summary. The adaptation of hamster modified equine abortion virus to the chick embryo is reported. Characteristic intranuclear inclusions were identified in the liver parenchymal cells of chick embryos and hamsters receiving serial passage chick embryo liver. Identification of the virus is substantiated by the use of the complement-fixation reaction.

1. Doll, E. R., and Wallace, M. E., *Cornell Vet.*, 1954, v44, 453.

2. Randall, C. C., unpublished data.

3. Doll, E. R., Richards, M. S., and Wallace,

M. E., *Cornell Vet.*, 1953, v43, 551.

4. Randall, C. C., McVickar, D. L., and Doll, E. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 465.

5. Doll, E. R., McCollum, W. H., Wallace, M. E., Bryans, J. T., and Richards, M. S., *Am. J. Vet. Res.*, 1953, v14, 40.

6. Randall, C. C., Ryden, F. W., Doll, E. R., and Schell, F. S., *Am. J. Path.*, 1953, v29, 139.

7. Doll, E. R., personal communication.

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Determination of Specific Activity of Sodium in Bone.* (21974)

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Studies of the role of skeletal sodium have been hampered by technical difficulties encountered in the analysis of this tissue for sodium content. These difficulties arise largely from the fact that bone contains tremendous amounts of calcium, the molar Ca/Na ratio being of the order of 25/1. This quantity of calcium causes serious interference with both the flame photometric and gravimetric methods for determination of sodium; and phosphate, in the quantities present in bone, may also interfere. Radioactivity measurements require large corrections for self-absorption if unaltered solutions of bone ash are used, and if attempts are made to precipitate calcium and phosphorus, as much as 20% of the radiosodium present may be removed by co-precipitation(1).

In an attempt to circumvent the difficulties inherent in analysis of bone for sodium, a new method was developed in which interfering substances are removed by cation exchange chromatography(2). Under the conditions employed, sodium appears in the eluate entirely free from phosphate, calcium, magnesium and potassium, whereupon the sodium-containing fraction can be easily analyzed by flame photometry.

The present report extends this investigation to include the behavior of radioactive sodium on the column, and further defines the accuracy and limitations of the method.

Method. Column characteristics — A column 1.3 x 60 cm is employed, containing 27 g (oven-dried basis) of the sulfonated polystyrene resin Dowex 50-x12 (50-100 mesh). The resin rests on a plug of Pyrex glass wool and fills the column to a height of 42 cm. Details of the preparation and operation of the column and the collection of eluate fractions are given in a previous publication(2). *Analysis of Effluent Fractions.* Analyses for Na^{23} and for K and Mg were carried out by standard methods (for references see 2). Calcium was determined by the hexanitratocerate technic (3). For purposes of this study, a special hood was fitted to the Weichelsbaum-Varney flame photometer in order to exhaust radioactive fumes to the outside. Measurements of Na^{22}† were made by pipetting 2 ml samples of eluate into 1.5 x 4.5 cm vials, which were then counted directly in a well-type scintillation counter. In instances where the activity was low, the sample was first evaporated on a steam bath to the appropriate degree of concentration. A known aliquot of Na^{22}, appropriately diluted in NaCl solution, served as a standard. Decay corrections were obviated by counting the eluate fractions and the standard solution on the same day. The usual corrections for coincidence effects were applied. *Preparation of Bone Samples.* Bone is first dried and then ashed overnight at 525°C in a muffle furnace. The ash is dissolved in 3 N HCl, and the resulting solution boiled for 10 minutes. This solution is then placed on the column, any remaining dark particles having been removed by filtration through Pyrex glass wool, allowed to flow into the resin bed, and elution with 0.8 N HCl be-

* Supported by a grant from the Playtex Park Research Foundation

† Radiosodium^{22} was obtained from the Oak Ridge National Laboratories.

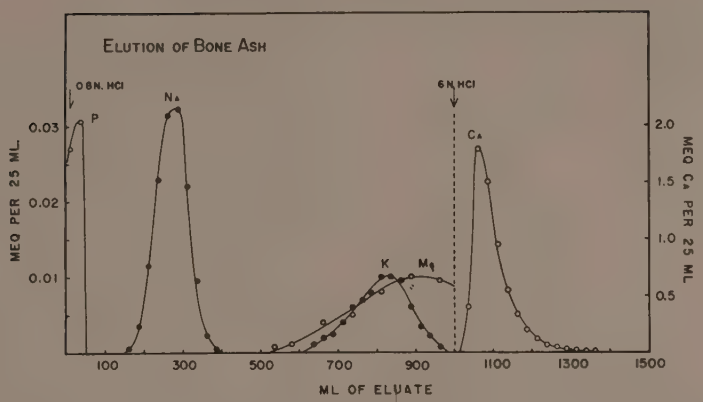

FIG. 1. Elution pattern of beef bone ash solution (0.5 g wet bone). Eluate collected in 25 ml fractions. Scale on the left-hand ordinate reads directly for Na and Mg, and should be multiplied by 0.1 for K. Phosphorus is plotted in mg $\times 10^{-3}$.

gun. Pyrex-brand glassware is used throughout.

Results. Elution of Bone Ash Solutions. A representative elution curve for a solution of bone ash (representing 0.5 g of wet beef bone)‡ is illustrated in Fig. 1. Phosphate is found only in the 0-50 ml range and K in the 600-1000 ml range. Magnesium first appears at the 525 ml mark and is not completely eluted by 1000 ml. Calcium does not appear by the 1000 ml mark, and requires 400 ml of 6 N HCl to effect a complete recovery. The 100-400 ml fraction which contains the sodium is thus free from P, K, Mg and Ca. No attempt was made to test for the presence of other ions.

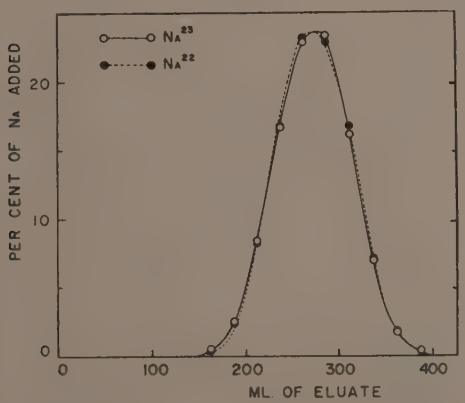

FIG. 2. Sodium elution pattern of beef bone ash solution (0.5 g wet bone) to which Na^{22} had been added. Eluate collected in 25 ml fractions. Eluriant 0.8 N HCl.

‡ The bone powder used was found by analysis to contain 12.2 meq. Ca and 0.25 meq. Na/g wet weight.

Fig. 2 illustrates the sodium-containing fraction of the eluate from a solution of bone ash to which a known amount of Na^{22} had been added. Both isotopes follow the same elution pattern with no appreciable change in the Na^{22}/Na^{23} ratio as the elution proceeds.

In Fig. 3 a series of elution curves for Na^{22} (approximately 0.4 μc in each instance) added to bone samples of varying size are depicted. The position of the elution curve remains fairly constant from one run to the next. However, it was found that the Mg curve shifted markedly with variation in sample size. Its position for 0.5 g of bone is indicated in Fig. 1. When 0.75 g of bone were used, Mg first appeared in the 400-425 ml fraction and with 1 g, it was present by the 300 ml mark. In a previous study(2), it was noted that as the sample size was increased, calcium appeared much earlier.

Recovery of Added Na^{22}. Recoveries of radiosodium added to solutions of bone ash and to a known quantity of NaCl are listed in Table I, the data indicating a high degree of accuracy for the procedure as we have employed it. In the instance in which NaCl was used, it was found that the specific activity of the sodium added to the column corresponded very closely to that recovered, the two values

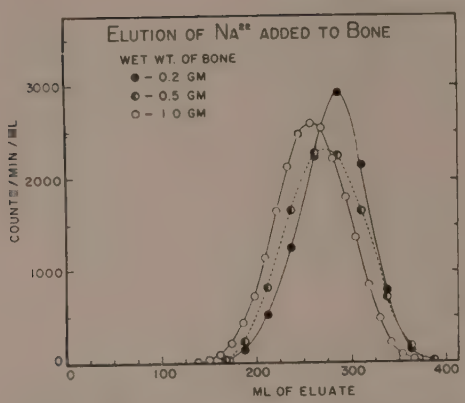

FIG. 3. Effect of sample size on sodium elution pattern. Elutriant 0.8 N HCl. Eluate collected in 12 ml or 25 ml fractions.

being 9.0 x 10^5 and 8.8 x 10^5 cts/min/meq. Na respectively.

Procedure adopted for determination of specific activity of bone samples. Bone is prepared as indicated above. The solution of bone ash is added to the column in a volume not to exceed 10 ml and allowed to flow into the resin bed. Elution with 0.8 N HCl is then carried out at a flow rate of 2.0 ± 0.5 ml per minute. The 0-100 ml eluate fraction is discarded. The 100-400 ml fraction is collected quantitatively and used for radioactivity measurements and for Na analysis by flame photometry. The elutriant is then changed to 6 N HCl in order to elute other cations more rapidly, and at least 400 ml is run through in order to completely elute K, Mg and Ca.

TABLE I. Recovery of Radiosodium.

Sample	Na^{22} added, cts/min.	Na^{22} recovered, cts/min.	% recovery
	× 1000		
NaCl, 0.3 mM	270.7	268.8	99.4
Bone, 1 g	263.9	261.6	99.2
1	289.9	291.9	100.7
1	288.1	293.2	101.8
1	255.0	254.9	99.9
1	251.9	253.9	100.7
.5	251.9	256.7	102.0
.2	252.8	256.3	101.4
.75	252.8	255.0	100.9
.5	244.5	246.6	100.8
		Avg	100.7%
		Stand. dev.	.94
		" error	.30

Approximately 300 ml of 0.8 N HCl are then run through the column in order to re-equilibrate the resin with the weaker acid. The resin is then agitated by repeated inversions of the column, whereupon it is ready for the next run. The entire procedure, including the reconditioning of the resin, occupies about 7 hours, during which time the column requires very little attention.

The Na-containing fraction of eluate is thoroughly mixed, and an aliquot evaporated to dryness on a steam bath in order to drive off most of the HCl. The residue is made up to volume with water and analyzed for Na^{23} by flame photometry. Depending on the amount of radioisotope present, radioactivity measurements are then made on another aliquot either in the original state, or after suitable concentration or dilution.

Discussion. It is evident that Na^{22} and Na^{23} behave in similar fashion on this cation exchange column, and that measurements of the specific activity of sodium in bone are feasible by this method. Interfering substances relative to Na^{23} analysis are removed by the procedure as we have employed it, and the necessity for large self-absorption corrections in Na^{22} analysis has been obviated since the Na-containing fraction of the eluate has a low solute content.

There are certain limits to sample size for a column of these dimensions. Analyses for Na^{23} gave distinctly less accurate results when amounts of the order of less than 0.06 meq. were added to the column(2), presumably because of the small but definite reagent blank. According to the results of our previous study on the composition of rat bone, sample size should therefore be not less than 0.5 g (wet weight) for very young animals and 0.25 g for adults(2). The upper limit depends on whether a slight degree of Mg contamination of the Na fraction can be tolerated; if not, the sample size should not exceed 1.5 g for young animals and 0.75 g for adults. The marked shift in the calcium curve places an absolute upper limit at 4 and 2 g, respectively, for the 2 age groups.

Since different batches of Dowex 50 may differ somewhat, elution curves should be run prior to using this method for analysis of bone

for sodium. In this connection it should be noted that the elution characteristics of the column we are now using differ slightly from those depicted previously(2).

Conclusion. A new method for the determination of specific activity of sodium in bone is presented. The method consists of placing a solution of bone ash on a cation exchange column. Elution with acid results in the quantitative recovery of both stable and radioactive sodium in a certain fraction of the eluate; this fraction is free from P, K, Mg and Ca and can be easily analyzed for both sodium and radiosodium.

The scintillation counter equipment was made available to us through the kindness of Mr. J. Russell Hayes of the Medical Division, Atomic Energy Project of the University of Rochester, and the specially adapted flame photometer by Dr. Taft Toribara of the Radiation Biology Division.

1. Davies, R. E., Kornberg, H. L., and Wilson, G. M., *Biochem. J.*, 1952, v52, xv.

2. Forbes, G. B., and D'Ambruso, Margaret, *J. Biol. Chem.*, 1955, v212, 655-661.

3. Kochakian, C. D., and Fox, R. Phyllis, *Indust. and Eng. Chem., Anal. Ed.*, 1944, v16, 762.

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Oestrogenic Activity of 16 Epi-Oestriol.*† (21975)

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The isolation of 16 epi-oestriol (oestra-1:3:5-triene-3:16B:17B-triol) from the acid hydrolyzed urine of pregnant women was described by Marrian and Bauld(1). Since the yield of this new Kober Chromagen was 0.1 mg/l and since this substance seems identical with 16 epi-oestriol as prepared by Huffman(2) considerable impetus was given to the present investigation in an effort to ascertain its activity on the growth potential of the uterus of the ovariectomized rat. Vaginal smears were also studied quite extensively. Moreover, since practically no biological information exists in the scientific literature on this naturally occurring oestrogen it seemed of particular significance at this time to elaborate its biological activity.

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§ The technical assistance of Miss Nancy M. Raney and Miss M. Claire Simoneau is gratefully acknowledged.

Procedures. Virgin, albino rats, approximately 100 days of age, were used in these experiments. They were started on treatment 7 days after bilateral ovariectomy. The 16 epi-oestriol was dissolved in U.S.P. propylene glycol, and injected subcutaneously once daily for 3 days. Vaginal smears were taken daily, and 72 hours after the first injection necropsies were performed. The uteri were removed quickly and slit longitudinally on bibulous paper, thus removing excess uterine luminal fluid. The uteri were then weighed to the nearest 0.2 mg on a Roller-Smith torsion balance. After the wet weights were determined, the uteri were placed in an oven at 110°C for dry weight determinations. A standard period of 24 hours at 110°C was used uniformly for drying each uterus. After this period the weight of each uterus was determined on a Roller-Smith torsion balance.

Results. A. Effect of 16 epi-oestriol on vagina of the ovariectomized rat. These results clearly indicate that 16 epi-oestriol has the ability to induce oestrogenic changes in the vagina of the ovariectomized rat (Table I). A demonstrable effect can be observed with 1.0 μg of this substance. Additional

TABLE I. Influence of 16 Epi-oestriol on Vagina of the Ovariectomized Rat.

Daily dose of 16 epi-oestriol, μg	Vaginal smear 24 hr after 1st inj.	48 hr after 1st inj.	56 hr after 1st inj.	72 hr after 1st inj.
.00 (1 cc propylene glycol)	L+++E	L++++	L++++	L++++
.01	L+++C+	L++E+C+	L++E+C+	L++E+C+
.1	"	L++C+	"	L+E+C+
1	L++E+C+	L+E+C+	L+E++C+	L+E+C++
5	L+C++	L+E++C+	"	LE++C++
10	L+EC++	LE+C++	L+EC++	LEC++
25	L+E+C++	"	"	LE+C++
30	"	L+C++	L+C+++	L+C+++
40	L+C+++	LC+++	LC+++	C++++
50	L+EC+++	LE+C++	"	LC++++
100	L+E+C++	"	"	"
.1 oestradiol 17β	L++E+	L+E+C++	L+EC++	C++++

L = Leucocytes; E = Nucleated epithelial cells; C = Cornified cells.
The pluses indicate the ratio of cell types found in the vaginal smear. For example: L+E+C++ = considerably more cornified cells than leucocytes and nucleated epithelial cells.

doses of 16 epi-oestriol produced a more definitive oestrous pattern, whereas 3 daily doses of 40 μg effected a full-blown estrous pattern in the vagina. Comparatively, this material is a very weak oestrogen, especially when one compares it with the vaginal cornifying effects of the various oestrogens(3,4). Of particular significance is the fact that 72 hours were required for the induction of a full vaginal oestrous smear (Table I).

B. Influence of 16 epi-oestriol on uterus of the ovariectomized rat. That this substance is a very weak oestrogen is further borne out by the information obtained from the wet and dry uterine weights. A slight, but statistically significant, increase in both the wet and dry uterine weights was observed in those animals receiving 1.0 μg of this material (Table II). A further increase in uterine weight was observed in the group of rats given 10 μg, and the maximum response was observed when 40 μg were administered daily for 3 days. Interestingly enough, parallel results were obtained with the various dosages of 16 epi-oestriol for both the vaginae and uteri (Tables I and II).

Included in Table II is the effect of injections of oestradiol 17β on the uterus of the ovariectomized rat. A dramatic uterine response, far above that obtained with 400 times (on a weight relationship basis) the amount of 16 epi-oestriol, was achieved with oestradiol. The increase was quite marked regardless of the vehicle used, sesame oil or propylene glycol (Table II).

Discussion. These results show quite conclusively that 16 epi-oestriol is the weakest of all known naturally occurring oestrogens described thus far(3-5). The exact function of this oestrogen is not known at this time, but it would not be too conjectural to think of this substance as a possible link in the metabolism of the oestrogens. Furthermore, while the recent results of Hisaw, Velardo, and Goolsby (5) indicated that oestriol had the ability to markedly restrict the action of oestradiol 17β on the growth of the uterus of the ovariectomized rat, preliminary results at hand seem to suggest quite strongly that this compound (16 epi-oestriol) shares in common with oestriol the property of restricting the uterine growth promoting action of oestradiol 17β. Additional research on this phase of our work is now in progress.

Summary. The oestrogenic activity of 16 epi-oestriol was determined. Virgin, adult albino rats were ovariectomized, and 7 days later were put on experiments. A dose-response (0.01 to 100 μg) of this substance was determined utilizing the uterus and vagina as end points. The results from these experiments indicate that 1.0 μg of this oestrogen produces a slight effect on the vagina as well

TABLE II. Influence of 16 Epi-oestriol on Uterine Growth in Ovariectomized Rats.

Treatment	No. of animals	Uterine wet wt (mg)	Uterine dry wt (mg)
Control, 10 full days after ovariectomy	25	115.5 ± 3.3*	22.4 ± .65
.1 μg oestradiol 17β in Sesame oil	36	227.3 ± 6.1	42.3 ± 1.8
.1 " in prop. glycol	15	236.0 ± 1.0	44.4 ± 2.5
.01 16 epi-oestriol	8	143.0 ± 7.2	27.0 ± 2.1
.1	15	126.4 ± 5.2	27.2 ± 1.5
1	8	150.0 ± 8.6	26.1 ± .8
5	8	142.7 ± 6.8	25.8 ± 1.7
10	9	171.4 ± 8.7	28.5 ± 1.6
25	9	164.3 ± 5.1	30.5 ± 1.1
30	7	167.2 ± 8.3	32.8 ± 2.0
40	7	181.6 ± 8.0	33.0 ± 2.0
50	7	175.1 ± 8.1	32.6 ± 1.9
100	6	180.2 ± 7.3	30.6 ± 1.5

* Avg ± stand. error.

as on the uterus. Three daily doses of 40.0 μg of 16 epi-oestriol produced a maximal effect on the vagina (full cornification) and the uterine weight. This substance seems to be the weakest of all the known naturally-occurring oestrogens described thus far.

1. Marrian, G. F., and Bauld, W. S., *Biochem. J.*, 1955, v59, 136.

2. Huffman, M. N., *J. Amer. Chem. Soc.*, 1942, v64, 2235.

3. Sondern, C. W., and Sealey, J. L., *Endocrinology*, 1940, v27, 670.

4. Sealey, J. L., and Sondern, C. W., *ibid.*, 1941, v29, 356.

5. Hisaw, F. L., Velardo, J. T., and Goolsby, C. M., *J. Clin. Endo. and Metab.*, 1954, v14, 1134.

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Pentose Dependence of *Lactobacillus gayonii* and Related Species for Early Growth.* (21976)

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Requirements for early growth of *Lactobacillus gayonii* and the closely related species, *buchneri, lycopersici, fermenti, mannitopoeus* and *brevis* have been investigated recently in the authors' laboratory because preliminary screening experiments showed this group of organisms to be particularly well suited for studies of D-α-hydroxy fatty acid and related lipid metabolism. The latter investigations, which were prompted by the finding that D-α-hydroxy fatty acids are essential in the nutrition of *Lactobacillus casei* 280-16(1,2), are to be described in subsequent reports. The effects of pentoses were studied because it had been shown previously that these sugars promote better growth and acid production than does glucose for this particular group of microorganisms(3), and it has now been found that although glucose may be utilized adaptively, an appropriate pentose is essential for early growth of most of these species.

Methods. The sugars tested were D-glucose (C.P. dextrose, J. T. Baker Chemical Co.), L-arabinose (Pfanstiehl Chemical Co.), D-arabinose, L-xylose, D-xylose, and D-ribose (products of Nutritional Biochemicals Corp.). Sugar solutions of appropriate concentration were added to 13 x 100 mm soft glass test tubes together with sufficient water to make 1.0 ml

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per tube and sterilized in the autoclave. The test medium, consisting of enzymatically digested casein (N-Z-Case, Sheffield Farms Co.) 0.6%, yeast extract (Difco) 0.2%, ammonia-hydrolysed yeast nucleic acid(4) 0.02%, glucose 0.5%, vitamin-mineral mixture(5) 20.6 mg %, and buffer solution(5) 5.0 ml %, was made to two-thirds final volume, autoclaved 15 minutes at 15 lb and added (2 ml per tube) aseptically to the previously sterilized tubes. The test microorganisms, described previously (6),† were maintained for the 10 years preceding the present experiments by serial subculture at 3-week intervals in Bacto-tomato juice agar (Difco) (the fresh stabs were incubated 40 hours then stored in a refrigerator). An inoculum medium was prepared by making a uniform suspension of dehydrated Bacto-tomato juice agar in the amount of water (cold) recommended by the manufacturer, stirring it for 10 minutes and then filtering to remove the agar, which, under these conditions, remained mostly undissolved. Fifteen ml aliquots of the filtrate were distributed into 50-ml centrifuge tubes and sterilized. Eighteen- to 24-hour cultures of the test organisms in this medium were centrifuged and resuspended in sufficient sterile saline to yield approximately 10^8 cells per ml. One-tenth ml of suspension per tube was employed as the inoculum. The inoculated tubes were incubated in a precision constant temperature water bath at 35°C.

Results. Bacto-tomato juice agar was employed as the inoculum medium (agar removed) to avoid adaptations during growth of the inoculum which might not have already occurred during maintenance of the test microorganisms. It is of interest in this respect that the composition of Bacto-tomato juice agar (tomato juice solids 2%, Bacto-peptone 1%, Bacto-peptonized milk 1%, agar 1.1%) includes tomato juice as the sole source of utilizable carbohydrate for most of these organisms since lactose, presumably present in peptonized milk, is not utilized appreciably by this group with the exception of *L. fermenti*(3). No report has been found concerning the types of sugar present in tomatoes, but it seems likely that pentoses are present in Bacto-tomato juice agar since the test organisms, as will be shown further on, remain unadapted to glucose utilization. The total sugars present in tomatoes average about 3.4% according to Chatfield and Adams(7).

Yeast extract was included in the test medium since it has been shown previously to stimulate growth of lactic acid bacteria(3), and ammonia-hydrolysed yeast nucleic acid was employed as a nucleotide supplement, since the latter is known to stimulate growth of *L. gayonii*(4). The growth rates of *L. brevis, L. buchneri, L. gayonii* and *L. mannitopoeus* were determined in the test medium with L-arabinose as the sugar source with and without the yeast extract and ammonia-hydrolysed yeast nucleic acid, and the stimulatory effects of these supplements (data not shown) were found to be independent and additive except with *L. buchneri,* for which the ammonia-hydrolysed yeast nucleic acid appeared to be inert. The complete test medium (containing both supplements) was used in subsequent experiments unless otherwise specified.

Glucose (0.5%) was autoclaved with the medium in all of the tests (unless otherwise noted) since Rogers *et al.*(8) have shown that an unidentified substance essential for early growth of *L. gayonii* is formed in glucose containing media upon heating. The concentrations of the various sugars tested (including glucose) were in addition to this basal glucose level, but the test sugars were sterilized separately from the medium to avoid alteration through "browning".

The data shown in Fig. 1 illustrate that *L. brevis* (B), *L. gayonii* (G), and *L. mannitopoeus* (M) respond markedly to L-arabinose (a) in 16 hours, but negligibly to glucose (g) under these conditions. Similar effects were noted with *L. buchneri* and *L. lycopersici* (data not shown), but *L. fermenti* exhibited a considerable 16-hour response to glucose, exceeded only by that to D-xylose and D-ribose.

All test organisms responded ultimately to glucose, although *L. buchneri* was slowest in doing so. The data presented in Fig. 2 show

† The American Type Culture Collection No. of *L. brevis* is incorrectly given as 8257 in the reference cited. The correct number is 8287.

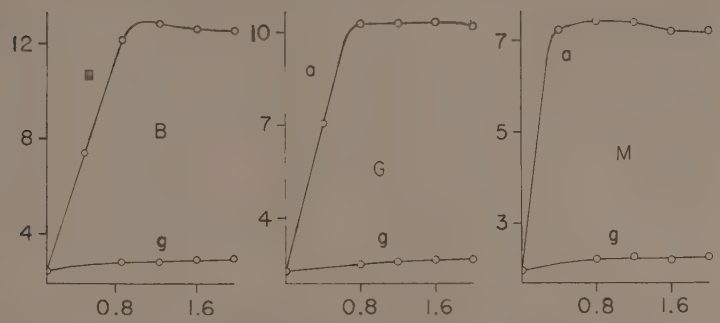

FIG. 1. 16 hr response of *L. brevis* (B), *L. gayonii* (G) and *L. mannitopoeus* (M) to L-arabinose (a) and D-glucose (g). Values on horizontal scales are sugar concentrations (%); those on vertical scales are titrations (calculated as ml of 0.01 *N* NaOH required to neutralize 1 ml of culture).

that the response of *L. buchneri* to glucose (g) was still only slight after 145 hours, whereas D-xylose (x) was nearly completely fermented in 145 hours and L-arabinose (a) in 50 hours.

Eighteen-hour responses of each of the test organisms to 1% L-arabinose, D-ribose, D-xylose and D-glucose, respectively, are given in Table I (the responses to D-arabinose and L-xylose were excluded from the table because they were negligible in all cases). L-Arabinose yielded the best or nearly the best response for all the test organisms except *L. fermenti*, and D-ribose and D-xylose were generally much better utilized than D-glucose (although some utilization of the latter was already in evidence).

FIG. 2. Growth of *L. buchneri* with 2% L-arabinose (a), 2% D-xylose (x), and 2% glucose (g). Values on horizontal scale are hours of incubation; those on vertical scale are titrations (calculated as ml of 0.01 *N* NaOH required to neutralize 1 ml of culture). The yeast extract, the ammonia-hydrolysed yeast nucleic acid and the initial supplement of glucose were omitted from the test medium in this experiment.

Results in the test medium solidified with agar and supplemented with D-glucose and L-arabinose, respectively, showed the same trends as in liquid medium. Colonies of *L. brevis*, *L. gayonii* and *L. mannitopoeus* in L-arabinose-supplemented agar medium were large (approximately 3 mm diameter) after 40 hours incubation under carbon dioxide, whereas those in D-glucose-supplemented agar medium were still nearly microscopic after this length of time. Colonies in the latter medium ultimately reached normal size, however, and the final number of colonies per unit volume of inoculum suspension were approximately the same with either D-glucose or L-arabinose, indicating that probably each cell in the inoculum suspension possessed an adaptive mechanism for the utilization of D-glucose.

The relatively large amounts of pentose required to promote growth of the test organisms (Fig. 1) suggest that the pentose functions as an energy source, rather than as a vitamin-like nutrient. A study of the enzyme systems involved has not been attempted, but a reasonable hypothesis appears to be that these pentose-requiring organisms possess a constitutive pentokinase together with an adaptive hexokinase. It seems unlikely that the growth-promoting effects of the pentoses were due to contamination with vitamin-like growth factors since the test medium contained yeast extract, which is generally found to be an adequate source of such unidentified nutrients. This possibility was rendered still less likely by the use of recrystallized L-arabinose, which

TABLE I. 18-Hour Response of the Test Organisms to 4 Sugars (at 1% Concentration).

	Growth response (acid production)*			
	L-Arabinose	D-Ribose	D-Xylose	D-Glucose
L. brevis	14.25	14.13	12.42	2.80
L. buchneri	10.28	4.87	4.80	.67
L. fermenti	2.38	9.81	8.17	5.33
L. gayonii	11.28	11.92	6.42	2.60
L. lycopersici	3.40	1.30	2.53	2.10
L. mannitopoeus	13.48	8.42	10.13	2.79

* Calculated as ml of 0.01 *N* NaOH required to neutralize 1 ml of culture.

was found to have retained all of its original growth-promoting activity.

That D-glucose has been employed with general success as a carbohydrate source for these and related microorganisms may be accounted for by adaption to D-glucose resulting under the test conditions employed. Cheldelin and Riggs(9), for example, carried their culture of *L. gayonii* (A.T.C.C. 8289) in a medium containing 1% glucose, 1% yeast extract, and 2% agar and, like most other researchers, employed an inoculum medium also containing glucose as the sole carbohydrate source. Such conditions would, of course, be certain to maintain this organism in its maximally adapted state.

Summary. Evidence has been presented indicating that an appropriate pentose is essential as an energy source for early growth of *L. brevis, L. buchneri, L. gayonii, L. lycopersici* and *L. mannitopoeus* if passage through a pentose-free glucose-containing medium, which would promote adaptive utilization of glucose, is avoided.

1. Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, 1953, v201, 621.

2. ———, *ibid.*, 1954, v211, 593.

3. Camien, M. N., Dunn, M. S., and Salle, A. J., *ibid.*, 1947, v168, 33.

4. Hutchings, B. L., Sloane, N. H., and Boggiano, E., *ibid.*, 1946, v162, 737.

5. Camien, M. N., and Dunn, M. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 177.

6. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H., *J. Biol. Chem.*, 1947, v168, 1.

7. Chatfield, C., and Adams, G., *U. S. Dept. Agr.*, 1931, *Circ. No.* 146.

8. Rogers, D., King, T. E., and Cheldelin, V. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 140.

9. Cheldelin, V. H., and Riggs, T. R., *Arch. Biochem.*, 1946, v10, 19.

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Effectiveness of Phenoxymethyl Penicillin V, and Sodium Penicillin G Against Hemolytic Streptococcus Infection in White Mice. (21977)

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The following experiments were conducted to determine the comparative effectiveness on a weight basis of phenoxymethyl penicillin V, and sodium penicillin G against hemolytic streptococcus infection in white mice.

Materials and methods. Two groups totaling 640 white mice in 2 tests were injected intraperitoneally with a 10^{-5} dilution of a 16-hour blood broth culture of hemolytic streptococcus C-203. This is usually somewhat more than 1000 LD_{50}, but less than 10,000 LD_{50}. In one test indicated below it was actually 4,050 LD_{50}, and in the other test it was 5,900 LD_{50} according to virulence tests which utilized 100 mice each. Following injection of the virulent culture, subgroups of 20 mice each were injected immediately by the subcutaneous route with either one or the other of the above penicillins. Different subgroups of 20 mice received either 7 or 9 2-fold

TABLE I. CD_{50}* of Two Penicillins in Hemolytic Streptococcus Infected Mice.

Penicillin used	1 dose therapy (mg)	2 dose therapy (mg)
Phenoxymethyl penicillin V	.207	.031
Penicillin G	.183	.030

CD_{50} computed by the method of Reed and Muench(1).

differing penicillin dosages in the range of 0.5 mg to 0.00095 mg. Stock solutions of the penicillins were furnished and prepared just before use in the mice by Dr. M. D. Bray of these laboratories. In one comparative test of the 2 penicillins, the mice got a single dose of penicillin, given at the time of infection. In the second test, a second dose of penicillin was given 5 hours after the first dose.

Results. As shown in Table I, a CD_{50} (dose curing 50% of mice) is indicated for the 2 penicillins from the results of these two tests. There was no significant difference in the therapeutic efficacy of phenoxymethyl penicillin V and penicillin G in the subcutaneous treatment of hemolytic streptococcus infections in white mice. The CD_{50} doses of the two were comparable, whether on single or double injection 5 hours apart. Furthermore the death rate in mice injected with subcurative doses of these two penicillins did not differ significantly.

Conclusion. Phenoxymethyl penicillin V and penicillin G have essentially the same curative properties against hemolytic streptococcus infections in white mice when these antibiotics are injected subcutaneously.

1. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

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Hemolysin Production in Irradiated Mice Given Spleen or Bone-Marrow Homogenate. (21978)

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Antibody formation is inhibited by exposure of the whole body to ionizing radiation (1,2), and the degree of inhibition is correlated with the time of antigen administration referred to the time of radiation exposure (3,4). Spleen or appendix shielding in rabbits during exposure to midlethal amounts of X-radiation resulted in a marked retention of the capacity to produce antibody to sheep erythrocytes(5). Preparatory treatment of the antigen with tissues or extracts of cells has recently been shown to improve the immune response in irradiated rabbits(6,7). Treatment of radiation injury in mice with spleen shielding(8) and spleen or bone marrow homogenates(9,10) accelerates hematopoietic recovery, lowers radiation mortality and has been shown to aid in the defenses against experimental infection(11). The present work was undertaken to study the hemolysin response in irradiated mice and to see whether the injection of marrow or spleen homogenate would hasten the recovery of antibody production following exposure to X-rays.

Methods. Mice, males of an inbred NIH strain, were 11 to 13 weeks of age at the time of irradiation. Mice of another strain. (BALB/cAn X $DBA_2J)F_1$, hereafter referred to as BALB/c, were used in one experiment. Littermates and treatment groups were distributed as nearly uniformly as possible within each group of 10 mice during the radiation exposure. Irradiation was carried out with a 200 KVP X-ray unit operating at 20 ma, with 0.25 mm Cu and 0.51 mm Al added filtration. HVL equal to 0.76 mm Cu. Radiation dose was 450 r (LD_4, 28 days) and in one experiment, 525 r, given at about 55 r per minute. Within an hour or 2 after irradiation, groups

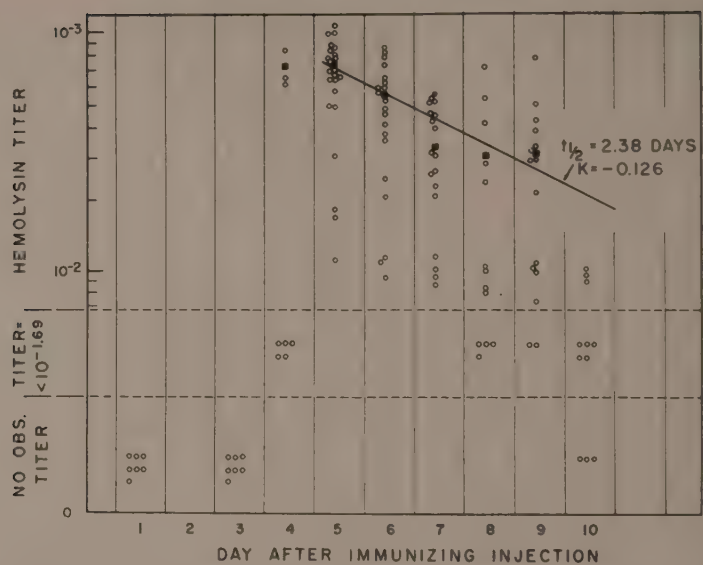

FIG. 1. Serum hemolysin titers of individual mice (○) sacrificed during the 10 day period following a single immunizing injection of sheep erythrocytes. Mean values for titers above 1:50 ($10^{-1.69}$) are denoted by (■), while titers below 1:50 or serums containing no observed hemolysin are shown schematically.

of the mice were given injections of suspensions of homologous spleen or bone-marrow from 4-week-old donor mice. Tissue, ground in a Potter type homogenizer and suspended in Tyrode's solution was injected intravenously. Each mouse received 0.5 ml of the suspension adjusted to contain the amount of tissue recoverable from the spleen or both femoral marrows of a single donor animal.

Mice were immunized with a single injection of a 2.5% suspension of washed sheep erythrocytes given intravenously at the rate of 0.01 ml per gram of body weight. This volume contained approximately 3.7 x 10^6 sheep erythrocytes which, from preliminary trials, was the lowest concentration that would yield satisfactory hemolysin titers in control mice. Serum from the individual mice collected at sacrifice was glycerinized and stored at about 3°C until the titrations were done within 3 to 4 weeks after the collection. Serum hemolysin content was estimated colorimetrically for individual mice following the method described by Taliaferro(12). Preliminary experiments had to be done to determine both the amount of the peak titer and the day upon which it would occur after a single immunizing injection. Experiments were then carried out to compare the peak serum hemolysin titers of irradiated homogenate-treated mice with their irradiated controls. In these experiments serum was collected on the 5th day after immunization from mice which had received their immunizing injection during the first or subsequent weeks through the 7th week after exposure to 450 r.

The possibility that the induction phase for the production of hemolysin might have been lengthened as a result of irradiation was tested in the irradiated homogenate-treated mice and their irradiated controls in other experiments. Serums of mice which had received their immunizing injection on the 4th, 5th, 6th or 7th week following exposure to 450 r were collected for hemolysin titration on the 5th or 6th through the 9th day after immunization.

Results. Initial phases of hemolysin production following a single injection of sheep erythrocytes are illustrated in Fig. 1, in which the titers are shown as the negative logarithms of the dilution per ml of the serum required

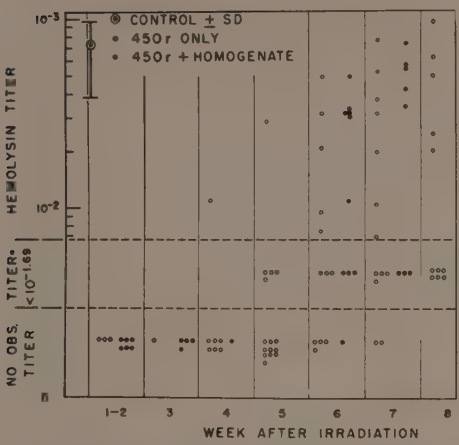

FIG. 2. Serum hemolysin titers on 5th day after immunization in marrow or spleen homogenate-treated irradiated mice compared with their irradiated controls. The mice were given a single immunizing injection of sheep erythrocytes during the indicated week following X-ray exposure and were sacrificed for the collection of their serums 5 days later.

to produce 50% hemolysis in a standard suspension of sheep erythrocytes. Serum samples in which the hemolysin content was just detectable, *i.e.* <1:50 or not detectable by our procedures, have been represented schematically in Fig. 1 and succeeding figures. The highest mean titer of the series, –2.73, corresponding to a dilution of 1:537 ± 333 (S.D.) per ml, occurred on the 5th day for serums of 23 mice. The slope of the line fitted by least squares through the means of titers above 1:50 for days 5 through 10 has the value –0.126. If it is assumed that group results reflect the changes in circulating hemolysin for any one animal, this slope represents a half life of about 2.4 days for the antibody.

Hemolysin titers on the 5th immunization day in spleen or marrow homogenate-treated mice are compared with their irradiated littermate controls in Fig. 2. Since no difference in hemolysin response was observed between the irradiated mice treated with bone marrow and those treated with spleen homogenate, the titrations of serums following these two treatments are given as for homogenate treatment. A gradual recovery of the immune response began during the 4th week after exposure to 450 r and continued at least into the 7th week (Fig. 2). The peak hemolysin titers in homogenate-treated mice agree closely with the titers of their irradiated controls and no indication of an accelerated return of the immune response is seen to be associated with the treatment.

Serums of irradiated or irradiated homogenate-treated mice showed little or no evidence of hemolysin production until the 4th week following exposure to 450 r. The homogenate treatment stimulated the early recovery of hematopoiesis since total leucocyte counts averaged significantly higher at 11 days after irradiation in 10 marrow and 10 spleen-treated mice than they did in 7 irradiated control mice

TABLE I. Hemolysin Production in BALB/c Mice Given Marrow or Spleen Homogenate after Exposure to 525 r.

	No. mice per group	Week following day of X-ray 1.5	3	4	5
Controls					
Hemolysin ± S.E.*	2–4		387 ±39	236 ± 50	287
WBC† ± S.E.	4	14.5 ±3.8		12.0± 1	
Spleen wt (mg) ± S.E.‡	4		175 ± 7.0	215 ± 8.5	253 ± 48.7
525 r only					
Hemolysin	9–10		—§	60	<50
WBC	10	.410± .05	1.8± .4‖	5.8± .6¶	
Spleen wt	9–10		223 ±82	124 ±101.6	124 ± 12.3
525 r + homogenate					
Hemolysin	15–18		—§	56	60
WBC	19	7.6 ± .5	7.9± .4‖	8.5± .6¶	
Spleen wt	17–19		130 ± 8.4	208 ± 23.4	188 ± 21.9

* Dilution/ml of mouse serum giving 50% hemolysis in a standard suspension of sheep erythrocytes. † Total leucocytes × 10^3/mm^3. ‡ Spleen wt in mg at sacrifice. § No observed serum hemolysin. ‖ WBC on day 18. ¶ WBC on day 26.

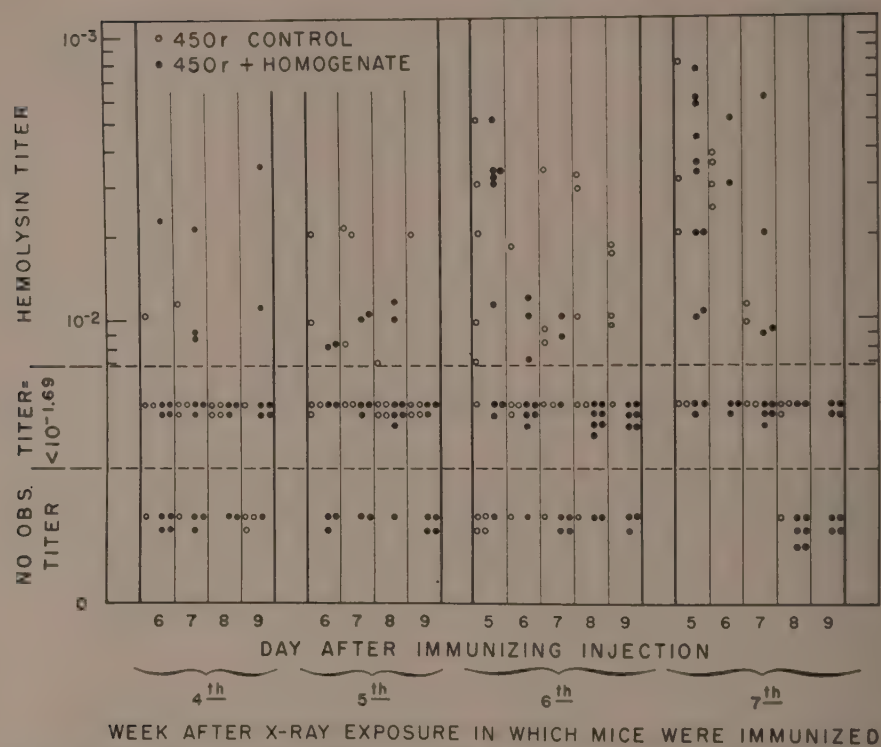

FIG. 3. Serum hemolysin titers in irradiated homogenate-treated mice and their irradiated controls for the indicated daily intervals during the 4th through 7th wk following exposure to 450 r.

(2145 ± 155, 2531 ± 591, 528 ± 110 cells per cu mm, respectively). Similar results were obtained in another experiment using BALB/c mice exposed to a somewhat higher radiation dose as shown in Table I. It is worthwhile to point out that the hyperplastic spleens seen in the third week (Table I) were not associated with improved hemolysin titers.

Fig. 3 summarizes the results of experiments which show that the time of maximum antibody production in the irradiated mice was not noticeably lengthened by the radiation exposure since there was no evidence that peak titers occurred later than the 5th day after immunization. The data of Fig. 3 indicate that the hemolysin titers tended to decline as the time from immunization increased, at least up to the 9th day. Furthermore, the homogenate treatment was without marked effect on the time of occurrence of maximum titer during the 4th through the 7th week after exposure to 450 r. The gradual recovery of the immune response following irradiation is shown in the higher individual titers and the relatively smaller proportion of low titered serums in the 7th week compared to the 4th and 5th weeks (Fig. 3).

Discussion. Homologous bone marrow and spleen cell homogenates, injected shortly after X-ray, fail to hasten the recovery of the production of anti-sheep erythrocyte antibody in irradiated mice. The favorable effect of the homogenate treatment on survival after exposure to X-ray doses in the lethal range is attributable in part at least to its salutary effect on the blood-forming tissues. The marrow and nearly every organ, as well as specific cell type, including lymphocytes and plasma cells, have been at various times considered the site of antibody formation(13). Although

homogenates of marrow or spleen either furnish cells which repopulate the hematopoietic tissue damaged by the radiation or supply a humoral substance which hastens the recovery of hematopoiesis(14), they produce no marked effect on the mechanism of hemolysin synthesis when given just after irradiation. Independent studies(15) of the cellular composition of the spleen and marrow of mice after exposure to 450 r have not shown unusual proportions of mature plasma cells either at 21 days when the spleens are enlarged or at 35 days when the antibody response has begun to recover.

More or less normal immune responses have recently been obtained in irradiated rabbits given antigen that had been incubated with lymph node cells(6) or minced spleen(7). In the experiments described in this paper (Fig. 2) antigen given as early as 2 days after the homogenate and within 4 days of irradiation did not result in a hemolysin response.

Summary and conclusions. 1. Recovery of the production of hemolysin in irradiated mice receiving bone marrow or spleen cell homogenate occurred at the same rate as in littermate control mice exposed to 450 r of X-rays. 2. Recovery of antibody production in response to injected sheep erythrocytes is gradual and begins in the 4th week after exposure of mice to 450 r. Recovery of the hemolysin response is not complete even at 7 weeks after this dose of radiation since many of the mice had peak serum hemolysin titers that were below the limits of non-irradiated controls. 3. The time required for the development of peak titer following a single immunizing injection was not lengthened by exposure to 450 r whole-body irradiation.

1. Hektoen, L., *J. Infect. Dis.*, 1915, v17, 415.

2. Taliaferro, W. H., and Taliaferro, L. G., *J. Immunol.*, 1951, v66, 181.

3. Hale, W. M., and Stoner, R. D., *Rad. Res.*, 1954, v1, 459.

4. Dixon, F. G., Talmage, D. W., and Maurer, P. W., *J. Immunol.*, 1952, v68, 693.

5. Jacobson, L. O., Robson, M. J., and Marks, E. K., PROC. SOC. EXP. BIOL. AND MED., 1950, v75 145.

6. Harris, T. N., Harris, S., and Farber, M. B., *Fed. Proc.*, 1954, v13, 496.

7. Jaroslow, B. N., and Taliaferro, W. H., Abstract of paper presented at 3rd Ann. Meet. of Rad. Res. Society, May 1955.

8. Jacobson, L. O., Simmons, E. L., Marks, E. K., Gaston, E. O., Robson, M. J., and Eldredge, J. H., *J. Lab. and Clin. Med.*, 1951, v37, 683.

9. Smith, W. W., Marston, R. Q., Ruth, H. J., and Cornfield, J., *Am. J. Physiol.*, 1954, v178, 288.

10. Lorenz, E., Congdon, C., and Uphoff, D., *Radiol.*, 1952, v58, 863.

11. Marston, R. Q., Ruth, H. J., and Smith, W. W., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 289.

12. Taliaferro, W. H., and Taliaferro, L. G., *J. Infect. Dis.*, 1950, v87, 37.

13. The Nature and Significance of the Antibody Response, Symposium No. 5, N. Y. Acad. Sci., Columbia Univ. Press, 1953, 13.

14. Congdon, C. C., and Lorenz, E., *Am. J. Physiol.*, 1954, v176, 297.

15. Grenan, Marie M., unpublished observations from this laboratory.

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Anticonvulsant Properties of Procaine, Cocaine, Adiphenine and Related Structures.* (21979)

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In low concentrations, local anesthetics block conduction without depolarizing nerves, by stabilizing neuronal membranes(1,2); but large doses given systemically can induce convulsions. It was therefore of interest that Yasukata(3) reported that procaine exhibits anticonvulsant effects in rabbits. Among the numerous classes of chemicals capable of preventing experimentally-induced and/or clinical seizures, there are several antihistaminic agents(4-6), ethers of phenylacetic and phenylbutylic acids(7,8), and other closely related substances. For these reasons, it seemed worthwhile to attempt to disclose some effective structural denominator of anticonvulsant activity among compounds in which dialkylaminoalkyl is linked via O, H or C to an aryl or aralkyl radical. To initiate this study, procaine and adiphenine (Trasentine) were tested for anticonvulsant properties in mice and compared with certain of their chemical and pharmacological congeners, particularly cocaine. The results obtained provide the basis for this report.

Methods. Male albino mice (CF #1 strain) obtained from the Carworth Farm were used as experimental animals. They were maintained on Purina Laboratory Chow and allowed free access to food and water except during the short time they were removed from their cages for testing. Anticonvulsant potency (ED_{50}) was determined by the maximal electroshock seizure (MES) test (50 mA alternating current, 0.2-second stimulus duration, corneal electrodes), and neurotoxicity (TD_{50}) was determined on the basis of overt signs of minimal neurological deficit. The details of the MES test and the endpoints employed for determination of acute neurotoxicity in mice have been described in full elsewhere(9). In order to determine the peak time for anticonvulsant action, the estimated ED_{85} for each drug was administered intraperitoneally to several groups of 6 mice each. Each group was then subjected to the MES test after a different time interval and the number of animals protected (*i.e.*, failing to exhibit the tonic-extensor component) was recorded for each group. The results obtained were plotted on graph paper and the time of peak effect estimated from the plotted points. For the determination of the ED_{50} (or TD_{50}), groups of 10 mice were given various doses of the experimental drug and tested at the previously determined time of peak effect until at least 3 points were established between complete protection (or toxicity) and no protection (or toxicity). The results were then plotted on logarithmic probability paper and a regression line was visually fitted to the plotted points. From this plot of the data, the ED_{50} or TD_{50} was determined and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon(10). The drugs subjected to the MES and neurotoxicity tests were as follows: procaine hydrochloride, cocaine hydrochloride, adiphenine (Trasentine),‡ β-(o-benzylphenoxy)-α-diethylaminoethane (No. BL 338-22)§, 2-diethylaminoethyl-1-phenylcyclopentane-1-carboxylate hydrochloride (caramiphen, Parpanit), diethylaminoethanol, and diethyl-(2-hydroxyethyl)-methylammonium bromide-α-phenylcyclohexaneglycolate (Ba 5473, oxyphenonium, Antrenyl).‡ The drugs were administered intraperitoneally as aqueous solutions. All animals were observed for the effect of the drugs on

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‡ Supplied by Dr. Ernst Oppenheimer, Ciba Pharmaceutical Products.

§ Supplied by Dr. S. B. Binkley, Bristol Laboratories.

TABLE I. Neurotoxicity and Anti-electroshock Potency of Procaine, Cocaine, and Trasentine.*

Drug	Neurotoxicity (TD_{50}), mg/kg	Max electroshock seizure test† Effective dose (ED_{50}), mg/kg	Protective index (P.I.)
Procaine	105 (98-113)	45 (42-49)	2.3 (2.1-2.5)
Cocaine	32 (29-36)	17.5 (15-23)	1.8 (1.6-2.0)
Trasentine	123 (116-131)	62 (58-67)	2.0 (1.8-2.2)

* All values in parentheses indicate 95% confidence limits.
† 50 mA, 0.2-sec. alternating current, corneal electrodes.

postictal phenomena, the details of which have been described elsewhere(11,12). In addition, the ED_{50}, as determined by the MES test, and the TD_{50}, as determined by the neurotoxicity test, for procaine hydrochloride, cocaine hydrochloride, and Trasentine were administered to groups of 5 mice each and tested for ability to prevent the tonic-extensor component of maximal seizures induced by the rapid intravenous injection of 38 mg/kg of pentylenetetrazol (Metrazol) (MMS test). The details of the MMS test have been published in full elsewhere(13).

Results. Procaine, cocaine, and Trasentine proved to possess anticonvulsant action, but their antiseizure activity appeared and disappeared very quickly. The time of peak action was estimated to be 5 minutes; by 20 minutes little if any residual effect could be observed. Therefore, all tests were performed 5 minutes after drug administration.

The neurotoxicity and anti-electroshock potency for procaine, cocaine, and Trasentine are summarized in Table I from which it may be seen that cocaine is 3 to 4 times more toxic than procaine or Trasentine. Indeed, 32 mg kg of cocaine produced signs of minimal neurotoxicity in 50% of mice as compared to 105 mg/kg for procaine and 123 mg/kg for Trasentine. The initial signs of cocaine neurotoxicity were agitation ad increased excitability; larger doses caused ataxia and postural abnormalities. The initial signs of procaine and Trasentine neurotoxicity were postural abnormalities and abnormal gait, especially dragging of the hindlimbs; moderately intoxicated animals were unable to maintain equilibrium. Doses of procaine in excess of 120 mg/kg produced excitation.

All 3 agents were capable of preventing the hindleg tonic-extensor component of maximal electroshock seizures (MES). Cocaine was approximately 2½ times more potent than procaine in this regard, and 3½ times more potent than Trasentine. For example, 17.5 mg/kg of cocaine abolished the hindleg tonic-extensor component in 50% of mice, whereas 62 mg/kg of Trasentine were required to produce the same level of protection. Larger doses than those required to protect 50% of animals by the MES test not only abolished the tonic-flexor phase as well as the tonic-extensor phase, but also produced the "anticoma effect" in the postictal period (prevention of immediate postseizure depression). Approximately 50% of animals receiving 75, 27.5, and 80 mg/kg of procaine, cocaine, and Trasentine, respectively, manifested this anticoma effect.

The protective index, *i.e.*, the ratio between TD_{50} and ED_{50}, was remarkably similar for all 3 agents. For example, the P. I. for cocaine was 1.8, whereas that for procaine was 2.3 and that for Trasentine was intermediate to these two values. Thus, all three agents exhibited anticonvulsant activity in non-toxic doses.

The anti-Metrazol activity exerted by the TD_{50} and the ED_{50} of each of the three agents was measured by the MMS test. The results obtained are summarized in Table II from which it may be seen that all 3 agents have the ability to decrease the incidence of the

TABLE II. Anti-Metrazol Effect of Procaine, Cocaine, and Trasentine.

Drug	Dose, mg/kg	Prevention of tonic-extensor component	Mortality
Procaine	45 *	3/5	4/5
	105 †	5/5	5/5
Cocaine	17.5*	3/5	2/5
	32 †	3/5	3/5
Trasentine	62 *	2/5	4/5
	123 †	4/5	2/5

* ED_{50} as determined by MES test.
† TD_{50} " " " neurotoxicity test.

tonic-extensor component of maximal Metrazol seizures; however, the required doses frequently synergized with the convulsant drug to cause death. The clonic component of Metrazol-induced maximal seizures was not abolished even by toxic doses of the 3 agents.

Beta - (o-benzylphenoxy)-α-diethylaminoethane (No. BL 338-22) and Parpanit, the chemical structures of which comply with the prerequisites mentioned in the introduction, were also tested for neurotoxicity and for ability to modify the pattern of maximal electroshock seizures. Their time of peak anticonvulsant action was estimated to be 10 minutes; by 30 minutes little if any residual effect could be observed. Both compounds were anticonvulsant, but their protective indices were near unity. Thus, the ED_{50}s for No. BL 338-22 and Parpanit were 52 and 80 mg/kg, respectively; their P.I.s were 1.0 and 1.5, respectively. In contrast, diethylaminoethanol and Antrenyl, the chemical structures of which only partially comply with the previously described structural prerequisites, were ineffective by the MES test even in toxic doses.

Discussion. The present investigation demonstrates that anticonvulsant activity is embodied not only in procaine, as was first reported by Yasukata(3) in rabbits, but also in cocaine and Trasentine. Since procaine and cocaine in large toxic doses are convulsant, it is of particular interest that in non-toxic doses these drugs act as anticonvulsants.

The anticonvulsant action of procaine, cocaine and Trasentine is rapid in onset and brief in duration. Peak action was observed 5 minutes after drug administration; by twenty minutes all anticonvulsant effect had disappeared. This very rapid onset of anticonvulsant activity and the evanescent duration of action probably account for previous failures to demonstrate antiseizure activity in local anesthetics.

The 5 compounds which have within their chemical structure a dialkylaminoalkyl linked via O, N or C to an aryl or aralkyl radical were all found to possess anticonvulsant activity as measured by the MES test; in contrast, the diethylaminoethanol moiety alone and Antrenyl, a quaternary ammonium compound, were devoid of such activity. (The ability of prior administration of diethylaminoethanol, the hydrolytic product of procaine, to prevent procaine convulsions in experimental animals(14) is probably accounted for by competitive inhibition on the basis of similarity of chemical structure.) Although the above findings suggest that compounds within this chemical series share a common anticonvulsant nucleus, further studies will be necessary precisely to determine what specific structural elements are essential for antiseizure activity.

Procaine, cocaine, and Trasentine all exhibit anticonvulsant effects in the laboratory which are qualitatively similar to those produced by the therapeutically useful antiepileptic drugs. However, the potential clinical value of compounds of this class must be considered minimal because of the very short duration of action and relatively low protective indices. Nevertheless, the data presented suggest that a search for congeners with less fugitive action is warranted. It is of interest in this connection that Bernhard and Bohm (15) have demonstrated that intravenous injection of the local anesthetic lidocaine (Xylocaine), an aminoacyl amide, can modify electrically induced cortical discharges in cats and interrupt epileptic seizures in patients. Preliminary experiments in our laboratory indicate that this compound, in non-toxic doses, is highly effective in abolishing the tonic-extensor component of maximal electroshock seizures. Indeed, others(16) have observed that intravenous procaine paradoxically increases the threshold for electrically evoked cortical responses in rabbits, but causes repetition and increased voltage of such responses as well as widespread irradiation. Since it is likely that the small neurones in the brain are the earliest to be blocked after systemic administration of local anesthetics, the possibility exists that the seizures induced by this class of drugs may be due to selective depression of inhibitory systems(16).

Summary. Five compounds—procaine hydrochloride, cocaine hydrochloride, adiphenine (Trasentine), β-(o-benzylphenoxy)-α-diethylamino-ethane, and Parpanit—the chemical structures of which have a dialkylaminoalkyl

linked via O, N or C to an aryl or aralkyl radical, were tested in mice for neurotoxicity and for ability to modify maximal electroshock seizures. In addition, procaine, cocaine, and Trasentine were tested for ability to modify maximal Metrazol seizures and to prevent postictal depression (anticoma effect). For comparison, two compounds (diethylaminoethanol and Antrenyl), the chemical structures of which only partially comply with the above structural prerequisites, were tested only for neurotoxicity and for ability to modify maximal electroshock seizures. The results obtained may be summarized as follows: 1. Procaine, cocaine, and Trasentine prevent the tonic-extensor component of maximal electroshock seizures in non-toxic doses and exert an anticoma effect in doses near the toxic level. All 3 agents prevent the tonic-extensor component of maximal Metrazol seizures, but only in doses which frequently synergize with the convulsant to cause death. 2. All compounds tested, the chemical structure of which contains a dialkylaminoalkyl linked via O, N or C to an aryl or aralkyl radical, were found to exhibit anticonvulsant activity. In contrast, diethylaminoethanol and Antrenyl were devoid of anticonvulsant activity. 3. The data indicate that compounds within this series share a common anticonvulsant nucleus, but further studies will be necessary precisely to characterize its structural requirements. 4. The laboratory search for congeners with less transient anticonvulsant action and higher protective index is warranted.

1. Bishop, G. H., *J. Cell. and Comp. Physiol.*, 1932, v1, 177.

2. Bennett, A. L., and Chinburg, K. G., *J. Pharmacol. and Exp. Therap.*, 1946, v88, 72.

3. Yasukata, M., *J. Yonago M. Assn.*, 1955, v6, 72.

4. McGavack, T. H., Elias, H., and Boyd, L. J., *Am. J. M. Sc.*, 1947, v213, 418.

5. Churchill, J. A., and Gammon, G. D., *J.A.M.A.*, 1949, v141, 18.

6. Swinyard, E. A., Jolley, J. M., and Goodman, L. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 239.

7. Swinyard, E. A., Madsen, J. A., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1954, v111, 54.

8. Sakai, S., Mishima, O., and Yuasa, Y., *J. Yonago M. Assn.*, 1954, v5, 47.

9. Swinyard, E. A., Brown, W. C., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1952, v106, 319.

10. Litchfield, J. T., Jr., and Wilcoxon, F., *ibid*, 1949, v96, 99.

11. Frommel, E., and Radouco-Thomas, C., *Helv. Physiol. Pharmacol. Acta.*, 1953, v11, 270.

12. Tanaka, K., in press, 1955.

13. Goodman, L. S., Grewal, M. S., Brown, W. C., and Swinyard, E. A., *J. Pharmacol. and Exp. Therap.*, 1953, v108, 168.

14. Richards, R. K., and Kueter, K. E., *J. Pharmacol. and Exp. Therap.*, 1946, v87, 42.

15. Bernhard, C. G., and Bohm, E., *Experientia*, 1954, v10, 474.

16. Toman, J. E. P., and Davis, Jean P., *Pharmacol. Rev.*, 1949, v1, 425.

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Reserpine and Thyroid Function. (21980)

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The introduction of reserpine with its mild sedative effects into common clinical use raises the question of the influence of this agent on thyroid function. Reserpine is generally assumed to act upon certain medullary centers(1), but the experiments of McQueen *et al.*(2) and of Kuschke and Gruner(3) appear to indicate a direct peripheral action as well. Very little work has been reported in the literature which would indicate whether reserpine exerts any influence either directly on the thyroid gland, or, through the hypothalamic-pituitary-thyroid axis, upon thyroid function. Certain observations, particularly the reserpine-induced bradycardia(4) and the effect of reserpine upon weight gain in "constitutional leanness"(5) could indicate an anti-thyroid activity of the drug. Moreover, Kuschke and Gruner(3) have reported that in the rat the effect of parenterally administered thyroxine upon the BMR is prevented by the administration of reserpine in large doses. However, the two published reports on the effect of Rauwolfia preparations on thyroid function as measured by the basal metabolic rate agree in finding no consistent effect. Erban and collaborators(6) found no effects in rats with a number of commercial preparations, and Moyer and associates(7) found no consistent changes in a small series of patients. Moyer also studied his patients by means of a radioiodine uptake test and obtained no consistent changes due to 6 weeks' medication with several crude Rauwolfia preparations. Since many patients entering thyroid clinics give a history of treatment with Rauwolfia preparations, it was considered important to establish whether this drug exerted any appreciable influence upon any of the commonly used criteria of thyroid function. A study was therefore undertaken in 18 chronically ill patients receiving reserpine at a level commonly used for the reduction of blood pressure.

To reconcile our results with those of Kuschke and Gruner the effect of reserpine in very high doses on thyroid function was also studied in a small number of rats.

Methods. Eighteen male patients were included. Blood pressure was determined daily throughout the study period. Base line values were obtained for the following criteria of thyroid function: (a) Serum total cholesterol, determined by the method of Kanter, Goodman and Yarborough(8); (b) 24-hour thyroidal radioiodine uptake; (c) 24-hour urinary radioiodine excretion; (d) serum protein-bound stable iodine, determined by Barker's (9) method; and (e) the 24-hour thyroxine synthesis measured according to Morton(10). In addition, the effect of a standard dose of adrenaline upon the level of circulating eosinophiles was determined by Thorn's(11) method. After completion of the base-line studies all patients received reserpine (Serpasil-Ciba) at a level of 0.125 mg t.i.d. for 2 days. The dose was then raised to 0.25 mg t.i.d. for another 24 days. In 2 patients the dose had to be reduced to 0.125 mg t.i.d. again after 1 and 11 days at the higher level, respectively, because of side effects. On the last 2 days of drug administration the battery of thyroid function and Thorn tests were repeated.

In the animal experiment 6 male Sprague-Dawley rats were injected intravenously with 0.40 mg/kg of serpasil in solution 2 hours before tracer iodine administration, and then received 0.60 mg/kg by subcutaneous injection every 3 hours. Four control rats received similar volumes of the vehicle furnished by Ciba Pharmaceuticals at the same time. Twenty-four hour thyroidal radioiodine uptake was determined and serum hormonal iodine synthesis were measured in all animals by the methods of Morton(10) and of Ingbar (12)

* The Radioiodine used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

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TABLE I. Post-Serpasil Thyroid Parameters as % of Pre-Treatment Level with Standard Deviations.

Group	No.	Cholesterol	24-hr I^{131} uptake	PBI	Urinary excretion*	Tx/To†
Whole	18	101 ± 22	96 ± 54	112 ± 16	111 ± 43	92 ± 41
+++	8	102 ± 28	110 ± 73	113 ± 24	118 ± 50	99 ± 46
++	7	99 ± 16	90 ± 34	110 ± 8	99 ± 23	77 ± 34
±	3	101 ± 16	67 ± 6	115 ± 13	120 ± 73	135 ± 19

+++ = Decrease of 20% or more in the mean systolic blood pressure. ++ = Moderate decrease in blood pressure. ± = Doubtful effect on blood pressure.

* 24 hr urinary excretion of radioiodine.

† % of 24 hr serum I^{131} activity present as hormonal iodine.

Results. A. Effects on Thyroid Function. Table I summarizes the results obtained with our group of patients, most of whom were markedly hypertensive prior to therapy. All patients participating in this study were judged to be euthyroid initially from the PBI and radioiodine uptake values. However, the thyroxine synthesis values were below 2% in ⅔ of the patients, which represents the lower limit of the euthyroid range. On recalculating the data by Ingbar's method, 10 patients had serum radio-thyroxine levels below .008% of the administered dose per liter of plasma, and only one patient showed a value above .012% per liter. In previous studies we have found low thyroxine synthesis values in the presence of normal PBI and uptake values in patients with cardiac disease of long duration (13), and we believe this to be a consequence of chronic stress. In Table I the experimental group has also been subdivided according to the effect upon the blood pressure produced by reserpine. Since the blood pressure was quite labile in most of the subjects, the patients were grouped into three categories according to the mean drop in blood pressure produced, with +++ indicating a good response (>20% drop of the mean systolic pressure); ++ a fair one and ± a doubtful or insignificant change due to the drug. The variability of the results may be related to the hypertensive state of most of these patients, since equally varied and random changes were found in two hypertensive subjects who were merely observed during the experimental period without medication, and since similar variability of the I^{131} uptake and BMR have been reported by Moyer(7).

From the results it is clear that reserpine at the levels employed does not affect thyroid function significantly.

B. Effects on the Thorn test. The Thorn test with adrenaline is not universally accepted as a test of the functional state of the hypothalamic-pituitary-adrenal axis. However in healthy individuals a drop of more than 30% of the circulating eosinophiles is produced 4 hours after an injection of 0.2 mg of adrenaline subcutaneously. In only 3 patients out of 14 was a drop of less than 30% observed in the number of circulating eosinophiles before reserpine therapy. After administration of reserpine for 25 days, 11 out of 17 patients showed no significant eosinopenic response. While the Thorn test with adrenaline certainly is not a specific test for pituitary-adrenal function, the reversal of the test under the influence of reserpine is of interest. At least part of the eosinopenic effect of adrenaline is generally assumed to be mediated via effects on cerebral centers higher than the pituitary. Since reserpine depresses afferent impulses to certain subcortical centers(14) and lowers central sympathetic reactivity(15) the results obtained in this study agree with the generally accepted mode of action of reserpine. However, the mechanism by which adrenaline exerts its eosinopenic effect is so poorly understood that it is not permissible to speculate on this basis alone.

C. Effects of reserpine at high dose levels. Since Kuschke and Gruner had observed what appeared to be an antagonism between reserpine and thyroxine in the rat, it became of interest to investigate whether any anti-thyroid activity of reserpine might occur in this species. Employing the drug at low levels of .10 mg/kg/day, the results were similar to those

TABLE II. Thyroid Activity of Rats on High Doses of Serpasil.

Group	No.	24 hr uptake (%)	Tx/To	Tx, % AD/l*
Control	4	8.4 ± 2.1	57.2 ±10.0	.088±.018
Serpasil	6	23.3 ± 4.1	10.8 ± 5.2	.18 ±.07

* Serum thyroxine-I^{131} as % of administered I^{131} dose/l of serum.

obtained in man, with neither the 24-hour uptake nor the thyroxine synthesis significantly affected. It was then decided to use reserpine at a dose level which had been shown to raise significantly the arousal in the brain stem of the rat(16). Under the conditions specified the data in Table II were obtained. It is significant that the reserpine-treated animals showed the symptoms of hypotensive shock: nasal congestion, somnolence and ptosis, as well as diarrhea. There was an obvious interference with renal excretion of iodide: Serum inorganic radioiodide levels were 20 times those in the controls 24 hours after tracer administration. This explains both the higher thyroidal uptake and the higher thyroxine synthesis value as calculated by Ingbar's method. It is possible that poor absorption of peripherally administered thyroxine due to the reserpine-induced hypotension also explains Kuschke and Gruner's results.

Summary. Reserpine at the dose levels employed clinically for the treatment of hypertension does not affect thyroid function in man. The effects of large doses in the rat appear to be due to hypotensive effects and interference with the renal excretion of iodide.

1. Bhargava, K. P., and Borison, H. L., *Fed. Proc.*, 1955, v14, 319.

2. McQueen, E. G., Doyle, A. E., and Smirk, F. H., *Nature*, 1954, v174, 1015.

3. Kuschke, H. T., and Gruner, H., *Klin. Wochenschr.*, 1954, v32, 563.

4. Plummer, A. T., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W., *Ann. N. Y. Acad. Sci.*, 1954, v59, 8.

5. Genest, T., Adamkiewicz, L., Robillard, R., and Tremblay G., *Canad. Med. Assn. J.*, 1955, v72, 490.

6. Erban, W., Lindner, A., and Watschinger, B., *Sci. Pharm.*, 1954, v22, 145.

7. Ford, R. V., Livesay, W. R., Miller, S. I., and Moyer, J. H., *Med. Rec. and Ann. Texas*, 1953, v47, 608.

8. Kanter, S. L., Goodman, J. R., and Yarborough, J., *J. Lab. Clin. Med.*, 1952, v40, 303.

9. Barker, S. B., Humphrey, M. J., and Soley, M. H., *J. Clin. Invest.*, 1951, v30, 55.

10. Morton, M. E., *Calif. Med.*, 1953, v78, 277.

11. Recant, L., Hume, D. M., Forsham, P. H., and Thorn, G. W., *J. Clin. Endocrin.*, 1950, v10, 187.

12. Ingbar, S. H., Freinkel, N., Hoeprich, P. D, and Athens, J. W., *J. Clin. Invest.*, 1954, v33, 388.

13. Morton, M. E., Goodman, J. R., and Florsheim, W. H., unpublished results.

14. Meier, R., Bein, H. J., Gross, F., Tripod, J., and Tuchmann-Duplessis, H., *Comp. Rend. Acad. Sci.*, 1954, v238, 961.

15. Schneider, R. A., *Ann. N. Y. Acad. Sci.*, 1954, v61, 150.

16. Barraclough, C/A., and Sawyer, C. W., personal communication.

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Electrolyte and Water Composition of Muscle and Liver in Hereditary Obese-Hyperglycemic Syndrome of Mice.* (21981)

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(Introduced by A. Baird Hastings.)

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The hereditary obese-hyperglycemic syndrome of mice(1) has, among other characteristics, an insulin resistant hyperglycemia and glycosuria(2). In a small proportion of obese animals, the hyperglycemia is not present but can be immediately elicited by injection of a single dose of growth hormone to which obese animals are extremely sensitive (3). Non-obese animals show the usual resistance of rodents to the diabetogenic effect of growth hormone. The syndrome is transmitted as a Mendelian recessive trait which affects both sexes in a similar manner. A number of relationships have been recognized between potassium deficiency and carbohydrate metabolism. The abnormalities of glucose production and utilization observed in insulin deficient rat liver slices incubated at a potassium concentration optimal for glycogenesis were similar to those observed in normal slices incubated in a low potassium medium(4,5,6).‡ Although no abnormality of potassium concentration was demonstrated in the liver cells of intact insulin-deficient rats (8), hyperglycemia and depletion of liver glycogen have been reported in rats maintained on a potassium deficient diet(9). Fatty acid metabolism of rat liver slices was increased by a high potassium concentration in the incubation medium(10).

These observations suggested that tissue analyses for water, fat potassium, sodium and chloride of mice with the hereditary obese-hyperglycemic syndrome and the estimation of intracellular concentrations of potassium and sodium might be of interest.

Materials and methods. Six male and 3 female mice with the hereditary obese-hyperglycemic syndrome were used. Three male and 3 female litter-mates served as controls. After a 24-hr fast (water *ad lib.*) the animals were anesthetized by intraperitoneal injection of one per cent aqueous sodium amytal. A dose of 0.2 mg per gram gave satisfactory anesthesia in 15 minutes. One obese animal required 0.3 mg per g. Blood sugar levels (11, 12) were determined on 0.02 ml of blood obtained from the tail. Blood for measurement of hemoglobin concentration was obtained in a similar manner. Serum for chloride(13) and sodium(14) determinations was separated from blood which was aspirated from the right side of the heart and placed under oil. Samples of muscle from the thigh and calf were dissected as free from adipose tissue as possible. Liver samples were obtained using care to avoid the gall bladder. Both tissue samples were minced, dried, defatted and extracted with 0.75 N nitric acid(15). Duplicate samples were used to measure the blood content of the tissues(15). The chloride content of the extracts was determined by a modification of the colorimetric micro-method of Lowry and co-workers(13). The sodium and potassium contents were determined by flame photometry(14).

Results. In Tables I and II the data from obese and control animals have been compared, all values in the latter table being calculated on a blood-free-fat-free basis. The obese animals were significantly hyperglycemic when sampled in the fed state several days before being sacrificed. The serum chloride concentration was lower in the obese animals, but this would only partially account for the difference in liver chloride concentration observed. The dry fat-free weight of

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‡ The *in vivo* intracellular potassium concentration can be maintained when liver slices are incubated only by increasing the potassium concentration of the medium to a level far higher than that of extracellular fluid(7).

TABLE I. Mean Body Weight, Blood and Serum Values.

	No. used	Age (days)	Body wt (g)	Blood: Fasting sugar (mg %)	Blood: Normal sugar (mg %)	Serum: Na (mEq/l)	Serum: Cl (mEq/l)
Obese	9	133	62 ± 7	151 ± 33	218 ± 38	145.6 ± 1.9	101.0 ± 1.8
Control	6	137	28 ± 2	94 ± 17	106 ± 13	146.3 ± .8	106.0 ± .9
p value			<.01	>.1	<.05	>.1	<.05

Digits following the ± sign are stand. error of the mean.

muscle was higher per unit of wet fat-free tissue in the obese animals. The potassium content of muscle was significantly lower per unit of dry fat-free tissue in the obese mice.

In Table III a correction has been applied to the data for the increment of water injected with the sodium amytal used for anesthesia. Derived values have been calculated for the amount of extracellular water in muscle, assuming that all chloride was extracellular(15). This permitted calculation of the concentration of potassium and sodium in intracellular water. No significant differences in these derived data between the obese and control mice were observed. The column headed "$(H_2O)_E$ less adipose tissue" will be discussed later.

Derived values have been calculated for the amount of extracellular water in liver, assuming that all the sodium was extracellular(16). This permitted calculation of the concentration of potassium and chloride in intracellular water. Here, as in muscle, no significant differences between the two groups were observed.

Discussion. Correction of the data for the increment of water injected with sodium amytal was necessary before comparing obese with control mice (Table III) because this increment was proportionally greater in the obese animals. With one exception the water injected was proportional to body weight, but the obese group contained less water per unit of body weight than their controls. To apply such a correction the following relationship obtained on mice with the same abnormality by Mayer and Hagman was used(17):

Grams of Total Body Water = 12 + (Grams of Body Weight x 0.11). Data were also reported by these authors showing that 15 minutes after intraperitoneal injection of deuterium oxide, the volume of dilution for the isotope labeled water had reached 88% of its equilibrium volume. These findings permitted calculation of the approximate water content of cells and extracellular fluid prior to the injection of aqueous sodium amytal solution. Specifically, the original serum water and tissue water values were multiplied by the following factor:

$$\frac{\text{Total Body Water}}{\text{Total Body Water} + (\text{Injected Water} \times 0.88)}$$

Application of this factor to the data increased the difference between the two groups with re-

TABLE II. Mean Values for Muscle and Liver; Original Data. The values are reported per kilo of wet fat-free, blood-free tissue or of dry fat-free, blood-free tissue.

	Per kg wet fat-free tissue: Dry fat-free wt (kg)	Wt of fat (kg)	Cl (mEq)	Na (mEq)	K (mEq)	Per kg dry fat-free tissue: K (mEq)
Muscle:						
Obese	.244 ± .002	.202 ± .041	14.4 ± .7	23.9 ± 1.1	108.4 ± 1.5	445 ± 6
Control	.235 ± .002	.075 ± .011	15.7 ± .7	23.7 ± .9	111.3 ± .9	474 ± 6
p value	<.01	<.05	>.1	>.1	>.1	<.01
Liver:						
Obese	.267 ± .002	.145 ± .021	26.8 ± .9	35.0 ± 1.3	84.7 ± 1.4	318 ± 6
Control	.259 ± .006	.103 ± .019	31.6 ± 2.0	39.1 ± 2.6	85.5 ± 2.5	329 ± 6
p value	>.1	>.1	<.05	>.1	>.1	>.1

Digits following the ± sign are stand. error of the mean.

TABLE III. Mean Values for Muscle and Liver Corrected for Water Injected with Anesthetic. Values are reported per kilo of wet fat-free, blood-free tissue or of intracellular water.

	Per kg wet fat-free tissue					Per kg intracellular water		
	Dry fat-free wt (kg)	Wt of fat (kg)	$(H_2O)_E$ (kg)	$(H_2O)_E$ less adipose tissue (kg)	$(H_2O)_C$ (kg)	K_C (mEq)	Na_C (mEq)	Cl_C (mEq)
Muscle:								
Obese	.256 ± .002	.212 ± .043	.133 ± .008	.107 ± .007	.612 ± .008	183 ± 3	7.1 ± 1.1	
Control	.241 ± .002	.077 ± .011	.138 ± .006	.129 ± .006	.622 ± .006	182 ± 3	5.0 ± 1.1	
p value	<.01	>.01	>.1	<.05	>.1	>.1	>.1	
Liver:								
Obese	.279 ± .001	.152 ± .022	.228 ± .007		.493 ± .008	176 ± 3		5.3 ± 1.3
Control	.265 ± .007	.105 ± .019	.256 ± .018		.479 ± .012	179 ± 4		6.9 ± 1.3
p value	>.1	>.1	>.1		>.1	>.1		>.1

Digits following the ± sign are stand. error of the mean.
Symbols: $(H_2O)_E$ extracellular water; $(H_2O)_C$ intracellular water; K_C intracellular potassium; Na_C intracellular sodium; Cl_C intracellular chloride.

spect to the dry fat-free weight of both tissue (muscle "p value" <0.01, liver "p value" <0.1). The serum chloride difference recorded in Table I was halved when the correction was applied.

It was apparent on dissection and confirmed by data in Tables II and III that considerably more adipose tissue was mingled with striated muscle fibres in the obese than in the control muscle samples. Data have been collected by Lowry and Hastings showing that adipose tissue from rat omentum contained 83% fat and 10% extracellular water(18). Assuming similar findings for adipose tissue of the obese and control mice, values were derived for $(H_2O)_E$ of muscle fibres after deducting the fraction associated with adipose tissue in the muscle samples (Table III). This approximation of the "true" $(H_2O)_E$ for muscle fibres gave a significantly lower value in the obese mice. The potassium concentration per unit of dry fat-free muscle was 6% lower in the obese group (Table II). However, $(H_2O)_C$ per unit of dry fat-free muscle was 7% lower in the fat animals after correction for injected water. "$(H_2O)_E$ less Adipose Tissue" was 22% lower when expressed per unit of dry fat-free muscle. This dehydration occurred even though water was available throughout the fasting period.

Studies in man have shown that the hyperglycemia and glycosuria following hypertonic glucose infusion is associated with sodium diuresis and a lesser degree of potassium diuresis(19). One may presume that the same type of electrolyte losses occurred in the obese mice and that while in the fasting state, dehydration was necessary to preserve the osmolarity of body fluids.

Summary. 1. Samples of striated muscle and liver from fasted mice with the hereditary obese-hyperglycemic syndrome were analyzed for water, fat, potassium, sodium, and chloride. 2. Some degree of intracellular dehydration and a greater degree of extracellular dehydration was observed without change in concentration of intracellular potassium.

1. Mayer, J., *Physiol. Rev.*, 1953, v33, 472.

2. Mayer, J., Bates, M. W., and Dickie, M. M., *Science*, 1951, v113, 746.

3. Mayer, J., Andrus, S. B., and Silides, D. J.,

Endocrinology, 1953, v53, 572.

4. Hastings, A. B., Teng, C. T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, 1952, v194, 69.

5. Renold, A. E., Teng, C. T., Nesbett, F. B., and Hastings, A. B., *ibid.*, 1953, v204, 533.

6. Hastings, A. B., Renold, A. E., and Teng, C. T., *Trans. Assn. Am. Phys.*, 1953, v67, 129.

7. Flink, E. B., Hastings, A. B., and Lowry, J. K., *Am. J. Physiol.*, 1950, v163, 598.

8. Tobian, L., Morse, W. I., and Hastings, A. B., unpublished.

9. Gardner, L. I., Talbot, N. B., Cook, C. D., Berman, H., and Uribe, C. R., *J. Lab. and Clin. Med.*, 1950, v35, 592.

10. Geyer, R. P., Meadows, M. F., Marshall, L. D., and Gongaware, M. S., *J. Biol. Chem.*, 1953, v205, 81.

11. Nelson, N., *ibid.*, 1945, v160, 61.

12. Somogyi, M., *ibid.*, 1945, v160, 61.

13. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M-L., and Farr, A. L., *ibid.*, 1954, v207, 1.

14. Flame photometer, model 52A, modified. The Perkin-Elmer Corporation, Glenbrook, Conn.

15. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, 1942, v143, 257.

16. Lowry, O. H., Hastings, A. B., McCay, C. M., and Brown, A. N., *J. Gerontol.*, 1946, v1, 345.

17. Mayer, J., and Hagman, N. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 647.

18. Lowry, O. H., and Hastings, A. B., unpublished observations.

19. Seldin, D. W., and Tarail, R., *Am. J. Physiol.*, 1949, v159, 160.

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Local Effects on the Feather Papilla of Thyroxine and of Progesterone.* (21982)

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Shedding and renewal of plumage occurs in birds in well-defined association with sexual and seasonal events. Normally, the standing lifeless feather is gradually expelled from the mouth of the follicle as a new feather arises following an activation which is initiated in the resting papilla, an organ that uninterruptedly occupies the internal well of the follicle and which is the actual site of the externally manifest cyclical events. Seasonal inertia of the papilla is a familiar phenomenon in laboratories utilizing experimentally the regenerating plumage. Forcible removal of the standing feather causes a tearing of the apical tissue of the papilla with haemorrhage into the lumen of the follicle(1). The wound stimulus is sufficient to assure immediate activation of the papilla in the capon whereas in the cock and hen, regeneration usually fails outside of the environs of molt. We may speak at such times of a "quiescent papilla." The papilla has long been shown to respond by growth to oral or parenteral administration of thyroid substance(2; and many others). The dosage requisite for this reaction was less as the season of normal molt approached(3), showing the gradual course of natural changes which culminate in that event. Recently it has been established that progesterone, when given as a single intramuscular injection of a preparation having slow absorption from local depots (Reposito Progesterone), also will cause a general activation of the feather papillae in adult fowl(4). There was no hint in these experiments that progesterone action was here in any sense *via* a stimulation of the bird's thyroid; this was checked in the feather structure where excess thyroid has long been known to produce specific, readily recognized consequences(5; and many others). The similar end effects seem accordingly independently due to either progesterone or to thyroxine although some common intermediate participation is not ruled out.

This possibility suggested following the consequences of separate local applications of either of the two substances to the papilla:

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these observations are recorded here.

Methods. Adult hens bearing feather papillae in the various physiological states were used. The test substance was applied to an area of one side of the bird's body and as control the respective solvent was applied to an identically prepared site in the opposite location. Progesterone was administered as a single intradermal injection of 0.20 ml of a solution of 50 mg/ml in sesame oil or as a daily local application during 4 weeks of progesterone in ointment, 50 mg/g.† Thyroxine was prepared as a solution of 5 mg (Thyroxin crystals Squibb) in 1 ml distilled water plus 1 drop of a 4% sodium hydroxide solution. It was administered as a single intradermal injection of 0.2 ml or as a daily repeated local application in ointment during 4 weeks. One-half ml of the thyroxine solution was rubbed up in a small amount of a commercial vanishing-type cream, the procedure repeated as required. Progesterone in oil was also injected directly into the superior umbilicus of the large secondaries in the expectation that the cavity of the quill of the standing feather would provide a source of gradual supply to the underlying papilla. This proved negative. Previous experiments had shown thyroxine in the same situation to enter into the general circulation and this route to be unsuited to the purpose.

Results. Five birds were treated with progesterone in ointment and 5 with thyroxine in ointment; in 3 of each series, the application was made to simultaneously defeathered areas; in the other 2, application was to the skin spaces separating adjacent standing feather rows. No activation was elicited in the papilla in either circumstance. Thyroxine in some amounts, however, apparently penetrated the epidermal tissues as there were subsequently noted characteristic changes of pigmentation in regenerating feathers of distant location. Thyroxine was given by intradermal injection on 28 occasions, progesterone on 27 occasions. The birds used were prepared so as to afford the 3 classes of papillae to either test substance. Birds were defeathered in small areas to assure the additional stimulus of plucking; the immediately following injections were made between adjacent denuded follicles. In affecting the resting papilla, the site injected was between 2 rows of standing feathers. The birds considered to have quiescent papillae had been plucked about 2 months earlier and had not exhibited regeneration up to the date of this treatment. The data from these series are summarized in Table I. The compilations refer to the total number of feather germs which were regenerated in the immediate vicinity of the point of injection. Examination of Table I shows thyroxine in intradermal injection to effect local proliferation of all types of the papilla. Experimental activation in resting and quiescent states, where it is opposed to zero regeneration in the control side, could not be clearer. However, the augmentation of the stimulus brought to growth in preliminary defeathering is equally evident. The properties of progesterone in these respects are less distinct. As administered, progesterone was negative in the resting papilla and the records of the quiescent papilla must be discounted in view of the regeneration occurring in the control area. In defeathering, a greater number of regenerations occurred in progesterone treatment which may suggest an increasing importance as the seasonal inertia of the papilla lessens. It is evident that the papilla has an annual or biannual physiological cycle which may proceed at very different levels (*cf.* capon and sexes of fowl), and which is triggered to an external event, presumably some hormonal impulse or withdrawal. If

TABLE I. Local Action on Feather Papilla of Thyroxine and of Progesterone.

Treatment	Papilla state	No. trials	Feather germs regenerated (No.) Treated side	Control side
Thyroxine (i.d.)*	Defeathered	15	119	59
	Resting	10	33	0
	Quiescent	3	10	0
Progesterone (i.d.)	Defeathered	15	96	58
	Resting	8	0	0
	Quiescent	4	9	10

* Intradermal.

† Progesterone was supplied generously by the Research Department of Ciba Pharmaceutical Products, Summit, N. J.

further studies should definitely prove progesterone to be locally negative in the feather papilla, this would show its systemic action to be upon some intermediate link. Since the bird's own thyroid is apparently unaffected by progesterone in circumstances where the substance is adequate for growth induction in the papilla, that gland is probably not the participant despite the very evident experimental effectiveness. In these aspects, the newly discovered role of progesterone casts some doubt on the concept that surges of thyroidal activity initiate the normal events of molt and suggests a revised approach to an important problem.

Summary. The respective local actions upon the feather papilla of thyroxine and of progesterone were examined. Application was made by intradermal injection to the immediate vicinity of the resting, quiescent and plucking-stimulated papillae. Thyroxine was effective in causing proliferation in all 3 circumstances; progesterone proved negative with this technic in the resting and quiescent states; it may have exerted a slight augmentation in the third.

1. Lillie, F. R., and Juhn, M., *Physiol. Zool.*, 1932, v5, 124.

2. Larionov, W. Th., and Kusmina, N., *Biol. Zentralblatt*, 1931, v51, 81.

3. Van der Meulen, J. B., *Proc. 7th World's Poultry Congress*, 1939, p109.

4. Shaffner, C. S., *Science*, 1954, v120, 345.

5. Cole, L. J., and Reid, D. H., *J. Agr. Research*, 1924, v29, 285.

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Fate of Ascorbic Acid in Early Radiation Damage.* (21983)

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(Introduced by F. Schlenk.)
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There is a paucity of information regarding the role of ascorbic acid in radiation damage. In light of the reported involvement of ascorbic acid in the conversion of folic acid to citrovorum factor(1) and, thereby, in nucleic acid synthesis, its sensitivity *in vivo* to irradiation is of interest. Thus far, the study of ascorbic acid has been concerned chiefly with its changes in the adrenal gland(2). We have investigated a variety of rat tissues from the standpoint of the concentration and total organ content of ascorbic acid as well as organ weight after whole-body X-irradiation. These organs included cervical lymph nodes, thymus, liver, spleen, kidney, intestinal mucosa, testis, brown adipose tissue, muscle, adrenals, and brain. The results indicate a highly selective action of irradiation on tissue ascorbic acid.

Methods. Male Sprague-Dawley rats, weighing 125-200 g, receiving a stock Purina Chow diet *ad lib.*, were irradiated at a rate of 23 r/min with a 250-kvp X-ray machine at 15 ma with a 0.5 mm Cu plus a 1.0 mm Al filter and the HVL equal to 2.0 mm of Cu. The target distance was 34 inches from the table supporting the animals. At 4, 28, and 72 hours after irradiation, groups of 4 to 6 animals were sacrificed. The organs were dissected out, blotted, and weighed on a torsion balance. A 9-inch strip of small intestine was slit, washed, and scraped to provide a sample of intestinal mucosa. The tissues were homogenized in 10% trichloroacetic acid; all of the ascorbic acid was converted to the oxidized (dehydro) form and determined as the osazone by the method of Roe and Kuether (3). No differentiation of oxidized and reduced ascorbic acid was carried out. Typical control values and their variation are shown in Table I. The values for concentration, total ascorbic acid and organ weight for the experimental groups are reported as a percent of the control values.

* Work performed under the auspices of the U.S. Atomic Energy Commission.

TABLE I. Ascorbic Acid Concentration and Organ Weights of Normal and Chloretone-Treated Rats.*

Organ	Normal		Chloretone-treated†	
	Ascorbic acid, mg %	Organ wt, mg/g	Ascorbic acid, mg %	Organ wt, mg/g
Adrenals	311.6 ± 28.8	.21 ± .01	391.4 ± 16.9	.20 ± .00
Thymus	66.8 ± 3.2	4.02 ± .57	64.4 ± 2.3	2.92 ± .29
Spleen	49.7 ± 2.9	4.44 ± .16	51.5 ± 2.2	3.56 ± .39
Cervical lymph nodes	46.5 ± 2.0	.81 ± .06	60.5 ± 3.0	.93 ± .17
Brain	41.7 ± .2	9.18 ± .32	38.6 ± .9	8.13 ± .04
Intestinal mucosa, 9 in.	33.0 ± 2.7	2.03 ± .04	38.0 ± 3.1	1.84 ± .12
Left testis	29.9 ± .9	5.70 ± .16	28.5 ± 1.3	5.72 ± .63
Liver	27.2 ± .9	54.5 ± 4.0	36.7 ± 1.3	61.3 ± .2
Left kidney	16.0 ± .1	4.87 ± .17	19.3 ± 1.4	5.34 ± .24
Pancreas	6.5 ± .5	3.62 ± .46	6.7 ± .5	2.50 ± .04
Skeletal muscle	5.0 ± .3	—	4.6 ± .3	—
Brown adipose tissue	4.5 ± .4	1.68 ± .12	4.7 ± .5	1.18 ± .12

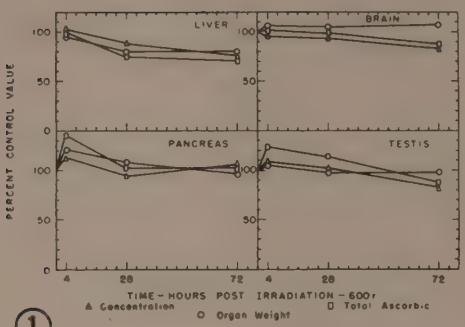
* Avg of 3 animals and its stand. dev.
† 1 mg of chloretone/ml drinking water for 6 days.

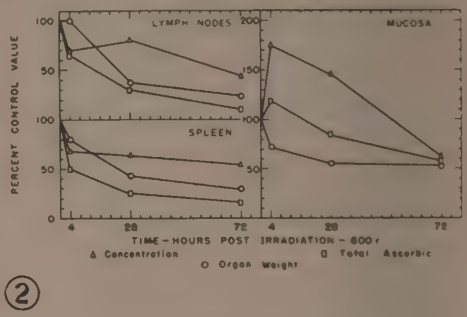
Results. After exposure to 600 r there were no gross changes in concentration or organ content of ascorbic acid or in organ weight in liver, brain, pancreas, testis (Fig. 1), muscle, kidney and brown adipose tissue. The adrenal glands showed an immediate drop in concentration at 4 hours, as has been noted by other workers(2); however at 28 hours the level had returned to normal and remained unchanged thereafter. The remaining 4 tissues: thymus, lymph nodes, spleen, and intestinal mucosa, underwent the rapid changes shown in Fig. 2. The total ascorbic acid content of lymph nodes, thymus, and spleen decreased to 30, 25, and 10%, respectively, of the original values. The concentration of ascorbic acid in these organs dropped abruptly within 4 hours to 65-70% and changed slowly thereafter. After 300 r, the changes in these organs were similar to those observed at the higher dose level while after 100 r the losses of ascorbic acid were small from the thymus and spleen and negligible from the lymph nodes.

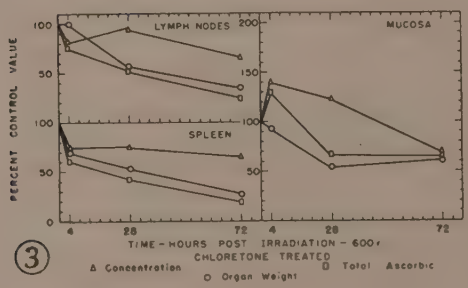
The changes observed in the intestinal mucosa 4 hours after X-irradiation were unique. Despite a loss of tissue weight, the total ascorbic acid content of this tissue had increased because of the concentration which had risen to 170% of the control value. This response likewise diminished slightly with lower doses of irradiation, but, even at 100 r, the concentration of ascorbic acid was 135% of the control value 4 hours after irradiation.

We investigated the water content of these four tissues after irradiation to determine, in the thymus, lymph nodes, and spleen, if there is an edema present which masks a loss of cells containing high concentrations of ascorbic acid, or in the mucosa, if there has been a dehydration of the tissue which caused an apparent increase in concentration. From Table II (which shows percent water content of organs of rats after exposure to 600 r), it can be seen that changes in water content of tissues could not account for the observed changes in ascorbic acid concentrations. In the lymph nodes there was even some dehydration.

The effect of stimulating synthesis of ascorbic acid on subsequent response to irradiation was next investigated. Administration of chloretone at a level of 20 mg/day for 7 days has been shown to result in urinary excretion of as much as 20-30 mg of ascorbic acid per day, in contrast to normal excretion of 0.5-2.0 mg/day(4). It does not necessarily follow, however, that concentration of ascorbic acid in tissues is correspondingly increased, since a low kidney threshold has been shown to exist in other species(5). That this appears to be the case may be seen by comparing the values for ascorbic acid concentration of tissues of rats given chloretone (1 mg/ml of drinking water) with those of untreated animals (Table I). Tissues of rats treated with chloretone exhibited, for example, the following increases in ascorbic acid concentration: liver, 35%;







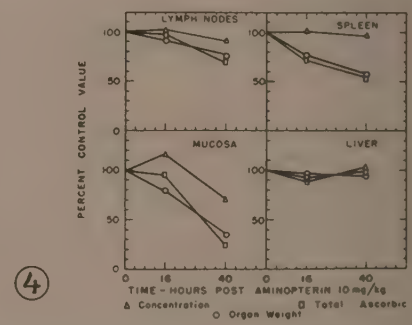


FIG. 1. Ascorbic acid changes in normal rat liver, brain, pancreas, and testis after 600 r.

FIG. 2. Changes in ascorbic acid in normal rat lymph nodes, spleen, and intestinal mucosa after 600 r.

FIG. 3. Ascorbic acid changes in chloretone-treated rat lymph nodes, spleen, and intestinal mucosa after 600 r.

FIG. 4. Changes in ascorbic acid in rat lymph nodes, spleen, intestinal mucosa, and liver after aminopterin treatment.

lymph nodes, 30%; intestinal mucosa, 15%; and spleen, 4%. These animals served as chloretone-treated, non-irradiated controls for the following irradiated animals. The remaining 24 rats were irradiated with 600 r and chloretone administration continued after irradiation. Their tissues displayed changes in concentration of ascorbic acid, total content and organ weight (Fig. 3) similar to those seen in previous experiments. The effect of chloretone was apparent in higher final levels of ascorbic acid in tissues of treated animals.

TABLE II. Organ Water Content.

Hr post-irradiation	% organ wt		
	Lymph	Spleen	Mucosa
0	86.8	78.1	81.7
4	76.9	76.0	80.7
28	79.5	77.3	82.0
72	77.8	75.5	82.8

and in a slight reduction of the rise in mucosal concentration. Administration of chloretone did not, however, abolish the decrease in concentration in lymphoid organs or stimulate recovery within 72 hours.

Alternatively, the effect of blocking the conversion of folic acid to citrovorum factor on the levels of ascorbic acid was studied. When aminopterin was administered intraperitoneally in doses of 10 mg/kg of body weight to a group of rats, the concentration and total content of ascorbic acid in lymph nodes, spleen, mucosa, and liver underwent the changes shown in Fig. 4. Aminopterin caused an involution of lymph nodes and spleen, but the loss of total ascorbic acid paralleled the loss in tissue weight, leaving the concentration unchanged. There was a small but significant rise in mucosal concentration after 10

hours, and no changes were observed in the liver.

Discussion. After irradiation with 600 r the majority of organs analysed presented no striking changes in ascorbic acid within 72 hours. However, organs of a lymphoid nature quickly dropped in both concentration and total content of ascorbic acid, while the mucosa increased in concentration during the same interval. Certain possibilities appear evident as explanations of the phenomena. For example, it is tempting to explain the rise in mucosal concentration and decreases in lymphoid tissue by means of a migration of lymphocytes to the intestinal epithelium. The rise in mucosal concentration after aminopterin treatment lends credence to this possibility, since such a migration has been observed after both aminopterin treatment and X-irradiation(6,7) and a histological examination of the small intestine confirms the presence of increased number of lymphocytes.†

The influx of lymphocytes cannot be wholly responsible for the findings because the increase in lymphocytes appeared to be insufficient to account for the total rise and the accumulation of such cells was not, apparently, matched by a corresponding loss of material from the lymphoid organs themselves.

The stability of ascorbic acid concentration in these organs after treatment with aminopterin would suggest that blocking conversion of folic acid to citrovorum factor does not in itself affect concentration of ascorbic acid. These data are, themselves, at variance with those of Williams(8) and Schwartz and Williams(9) who found a decrease in liver ascorbic acid concentration 13 days after feeding of aminopterin at a level of 4 mg/kilo of diet. This discrepancy may be due either to route of administration or to high dosage (LD_{100}—72 hours) employed in the present study which was, in our consideration, necessary to duplicate the acute aspect of irradiation. Even at this level, however, the concentration of ascorbic acid in tissues did not decrease within 40 hours.

Furthermore, the effect of stimulating the synthesis of ascorbic acid on changes in concentration or organ content of ascorbic acid after irradiation is negligible, suggesting that an impairment of the synthetic process does not seem to be responsible. Nor does the stimulation of ascorbic acid synthesis by chloretone seem to be abolished by irradiation.

This then focuses attention on the relationship of ascorbic acid to the particular tissue in which its concentration is so readily changed by X-ray. The observed rapid rise of ascorbic acid and the well known inhibition of mitosis in the intestinal mucosa after irradiation suggest that the accumulation of ascorbic acid is a result of arrested mitosis and that ascorbic acid is requisite to and/or consumed by mitotic activity. A similar suggestion has been made by Stern and Timonen (10) working with lily anthers. Failure to observe similar changes in the other tissues may be due to their relatively much lower mitotic activity. Coupled with this, the loss of ascorbic acid from lymphoid tissues may be explained by the known radiation-induced fragility of several cell types in relatively high concentration in these organs.

Summary. 1. There were no gross changes in concentration or total content of ascorbic acid within 72 hours after 600 r of X-ray in rat liver, brain, pancreas, testis, muscle, kidney, or brown adipose tissue. Thymus, lymph nodes, and spleen showed a drop in ascorbic acid concentration within four hours, and the content of ascorbic acid continued to drop throughout the period studied. Intestinal mucosa showed a sharp increase in ascorbic acid concentration during the first 4 hours followed by a gradual decline. 2. Stimulation of ascorbic acid synthesis by chloretone administration does not modify the effects of irradiation, nor does the hydration of these tissues change in a fashion which might account for the observed phenomena. 3. The observed changes in concentration could not be duplicated by the administration of aminopterin at a level

† We wish to thank Dr. F. Wassermann for preparation of the material and Dr. A. M. Brues and Mrs. Agnes Stroud for assistance in interpretation of the specimens.

of 10 mg/kg body weight.

1. Nichol, C. A., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 52.

2. Oster, H. L., Kretchmar, A. L., and Bethell, F. H., *ibid.*, 1953, v84, 470.

3. Roe, J. H., and Kuether, C. A., *J. Biol. Chem*, 1943, v147, 399.

4. Jackel, S. S., Mosbach, E. N., Burns, J. J., and King, C. G., *ibid.*, 1950, v186, 569.

5. Friedman, G. J., Sherry, S., and Ralli, E. P., *J. Clin. Invest.*, 1940, v19, 685.

6. Phillips, F. S., Thiersch, J. B., and Ferguson, F. C., *Ann. N. Y. Acad. Sci.*, 1950, v52, Art. 8, 1349.

7. Pierce, M., *Histopathology of Irradiation*, 1948. 502, W. A. Bloom, ed., McGraw-Hill, N. Y.

8. Williams, J. N., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 315.

9. Schwartz, M. A., and Williams, J. N., Jr., *J. Biol. Chem.*, 1952, v194, 711.

10. Stern, H., and Timonen, S., *J. Gen. Physiol.*, 1954, v38, 41.

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Relationship of Prolonged Drainage of Bile through Pancreatic Duct System to Pancreatitis.* (21984)

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The role of reflux of bile into the pancreatic duct system in the etiology of acute hemorrhagic pancreatic necrosis has been a subject of intensive investigation for many years. It is well known that when sterile bile is injected into the pancreatic duct of experimental animals, under certain conditions, a fulminating hemorrhagic pancreatic necrosis results quite regularly. However, there is a great deal of evidence that under other more physiologic conditions the presence of bile in the pancreatic duct is harmless to the pancreas. Mann and Giordano(1) found that, if the pressure exerted during retrograde injection of bile into the pancreatic duct were maintained within physiologic limits, no pancreatitis developed. It was shown in this laboratory(2) in cats that when the common bile duct was occluded distal to the entrance of the pancreatic duct so that bile could flow into the pancreatic duct under secretory pressure of the liver or pressure of the contracting gall bladder, pancreatitis developed in only a small proportion of animals, unless the cats were fed fatty meals supplemented with bile salts. Leven(3) and Colp and Doubilet(4) showed that reflux of iodized oil into the pancreatic duct could be demonstrated in cholangiograms in about 20% of patients with common bile duct drainage. This reflux was attributed in most cases to spasm of the sphincter of Oddi. Hicken and McAllister(5) using a water soluble contrast agent and very low injection pressures found that even in patients with no intraductal pathology, reflux of contrast material into the pancreatic duct could be demonstrated on routine cholangiograms in a high percentage of cases. Fisher *et al.*(6) drained the bile through the pancreatic duct by connecting a catheter in the common bile duct with a catheter in the pancreatic duct. In their 4 experiments the longest follow-up was 7 days. None of these animals developed any pancreatitis.

The experiments reported here, from August 1950 to July 1951, were designed to show the effect upon the pancreas of draining all of the bile secreted by the dog's liver through the pancreatic duct system over long periods of time.

Methods. The entire biliary flow in a series of dogs was caused to pass through the pancreatic duct into the duodenum by one of 3 operations. Type I: the duct of Santorini

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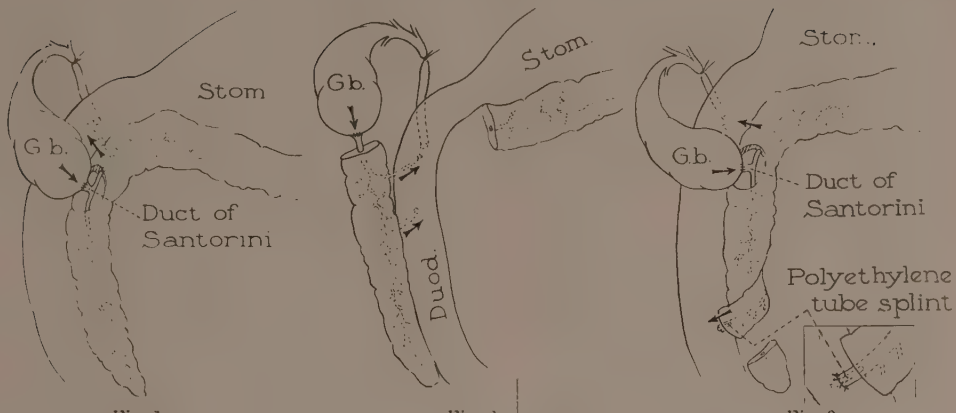

Fig. 1 Fig. 2 Fig. 3

FIG. 1, 2 and 3 show Type I, II and III operations, respectively. Bile drains through pancreatic duct system as indicated by arrows.

(the larger duct in the dog) was anastomosed to the gall bladder and the common bile duct was tied off. Under these circumstances, bile flows from the duct of Santorini into the duct of Wirsung through anastomoses between the two duct systems which are regularly present in the dog and thence into the intestine through the duct of Wirsung. Type II: the body of the pancreas was transected and the cut end of the pancreatic duct in this part of the pancreas was anastomosed to the gall bladder and the common bile duct was ligated. In this preparation bile flows from the gall bladder into the pancreatic duct through the anastomosis and reaches the intestine by way of the normal pancreatic duct orifices. Type III: the duct of Santorini was divided just proximal to its orifice in the duodenum, and this duct was anastomosed to the gall bladder. The distal end of the duodenal portion of the pancreas was transected and the cut end of the duct was located and anastomosed into the duodenum using a small polyethylene tube as a splint. The common bile duct was either ligated or anastomosed to the gall bladder. Bile flows into pancreas through the major duct and out into the intestine through the point where the duct is implanted into the duodenum. Serum amylase and serum bilirubin determinations were made on all dogs at weekly intervals.

Results. Of the 47 dogs used in these experiments, 18 had patent functional anastomoses between the gall bladder and the pancreatic duct system and between the pancreatic duct and intestine at the time that they died or were sacrificed, and these are the animals included in this study. Eleven of the remaining animals are not included because at the time of death it was found that the anastomosis between gall bladder and pancreatic duct or between pancreatic duct and intestine was not patent, and 18 were not included either because they did not survive for a significant period after their operation or because there was insufficient data on them.

Of the 18 dogs with intact anastomoses, only 3 had clinically significant degrees of pancreatitis, and only one of these had a typical full-blown pancreatic necrosis. The case of severe pancreatic necrosis occurred 22 days postoperatively in a dog in which a normal serum bilirubin and only a moderately elevated serum amylase had been noted only 4 days earlier. The other 2 dogs had only local areas of necrosis in the pancreas.

Fifteen of the 18 dogs with intact anastomoses did not develop significant degrees of pancreatitis. Eight were not jaundiced at the time of death, indicating that the bile was passing relatively unimpeded through the pancreatic duct system into the intestine. Four of these 8 dogs lived from 58 to 138 days, and

the average survival time for the group was 60 days. Gross inspection at autopsy showed the pancreas to be firm and atrophic; the gall bladder and bile ducts were dilated and the liver was firm and rough. Microscopic examination of the pancreas usually showed only mild degrees of interstitial pancreatitis and fibrosis.

The remaining 7 dogs with intact anastomoses had a significant degree of clinical jaundice at the time of death even though a patent channel was demonstrated from the gall bladder through the pancreatic duct system into the duodenum at autopsy. This circumstance suggests that although some bile was probably passing through the pancreatic duct system, the flow was not free enough to maintain the serum bilirubin at normal levels.

Summary and conclusions. 1. The entire flow of bile was successfully diverted through the pancreatic duct system into the intestine in a total of 11 dogs. Eight of these dogs did not develop any significant degree of pancreatitis even though the average observation period for this group was more than 60 days, and only one dog in the series developed a full-blown acute pancreatic necrosis. 2. The results of these experiments indicate that the presence of bile in the pancreatic duct system under physiologic pressures is tolerated for long periods of time without any serious damage to the pancreas in most cases.

1. Mann, F. C., and Giordano, A. S., *Arch. Surg.*, 1923, v6, 1.

2. Wangensteen, O. H., Leven, N. L., and Manson. M. N., *Arch. Surg.*, 1931, v23, 47.

3. Leven, N. L., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 808.

4. Colp, R., and Doubilet, H., *Surgery*, 1938, v4, 837.

5. Hicken, W. F., and McAllister, A. J., *Am. J. Surg.*, 1952, v83, 781.

6. Fisher, B., Fisher, E. R., and Selker, R., *Surgical Forum Clinical Congress, Am. Coll. of Surg.*, 1953, p406, Saunders, Philadelphia, 1954.

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Lactic Dehydrogenase Activity in Blood.* (21985)

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The observation that during experimental and clinical myocardial infarction glutamic oxaloacetic transaminase is released from cardiac muscle resulting in increased enzyme activity in the serum(1-5), suggested that other cardiac tissue enzymes behave similarly during myocardial infarction. Although present in other tissues in greater activity, lactic dehydrogenase, the enzyme concerned primarily with the reduction of pyruvic acid to lactic acid, is present in appreciable activity in cardiac musculature. In order to ascertain whether lactic dehydrogenase (hereafter referred to as LD) activity is increased in the serum during myocardial infarction, it was necessary to first demonstrate its presence in human and animal blood, and to delineate variations in LD activity in the blood of normal and diseased man.

Methods and materials. Lactic dehydrogenase is concerned with the reduction, in the presence of reduced diphosphonucleotide (DPNH), of alpha-keto and of alpha, gamma-diketo acids; maximum reduction by LD has been shown to occur with pyruvic acid. Inasmuch as LD catalyzes the reduction of various alpha-keto and alpha, gamma-diketo acids, the reduction of a substrate in the presence of LD cannot be used to specifically identify an alpha-keto or an alpha, gamma-diketo acid. However, the catalytic reduction of a known amount of a specific keto acid may be used to quantitatively estimate LD activity(6). The *LD activity* of human serum was measured

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spectrophotometrically. To 2.4 ml of pH 7.4 phosphate buffer, 0.1 m of serum and 0.1 m of reduced diphosphonucleotide (DPNH) (2.5 mg per ml) were added. After 20 minutes, 0.1 ml of sodium pyruvate (2.5 mg per ml) was added. The optical density at wavelength 340 mμ was followed for 3 to 5 minutes at intervals of 10, 15, or 30 seconds depending on the rate of reaction. The rate of decrease of optical density representing the rate of oxidation of DPNH was taken as the measure of LD activity of serum. The reaction was followed in a Beckman model DU spectrophotometer using a tungsten light source and at room temperature, 24 to 27°C. The activity is expressed as units per ml of serum per minute. One unit equals a decrease in optical density of 0.001 per minute per ml under the conditions described.

When DPNH and pH 7.4 phosphate buffer were added to serum without the addition of pyruvate, the optical density of the mixture decreased slowly for up to 20 minutes indicating the oxidation of a finite quantity of DPNH. This initial oxidation of DPNH by serum without added pyruvate appears to depend on the presence of alpha-keto and alpha, gamma-diketo acid in the serum (in the presence of serum LD). The reaction stopped when the keto and diketo acids were enzymatically reduced to lactate by DPNH. The addition of more DPNH will not restart the reaction, but the addition of pyruvate resulted in a steady decrease of optical density which was taken as a measure of LD activity of the serum. The optimal concentration of DPNH was found to be 0.1 ml (2.5 mg DPNH per ml in water made basic to pH 9.0 with sodium hydroxide) in a total reaction mixture of 3.0 ml (3.8×10^{-7} M). At pH 7.2 to 7.6, serum LD was optimally active and this is in substantial agreement with that reported for LD from other tissue sources(6-8). The LD activity of serum was maximal when 0.1 ml of pyruvate (2.5 mg per ml of distilled water) was used in a total reaction mixture of 3.0 ml (0.23×10^{-5} M). The rate of LD activity was directly proportional to the quantity of serum used. No activators of LD were used although it has been reported(9) that amino-DPNH potentiates LD activity. Competitive inhibitions of LD by alpha, gamma-diketo valerie acid has been reported(6) but the enzyme is insensitive to sulphydryl reagents(10). Serum samples stored from 30 minutes to 96 hours at room temperature or for periods of from 1 hour to 1 week in the refrigerator (0° to 5°C) showed no significant alteration in serum LD activity; the heat stability noted is in essential agreements with reports of LD derived from other sources(7, 11).

TABLE I. Distribution of Lactic Dehydrogenase Activity in Venous Serum of Normal Adults.

LD activity (units)	No. of individuals	%
250-290	7	4.3
300-340	18	11.2
350-390	25	15.5
400-440	32	20.0
450-490	22	13.7
500-540	16	10.0
550-590	17	10.6
600-640	7	4.3
650-690	7	4.3
700-740	2	1.2
750-790	4	2.5
800-840	3	1.9
850-890	1	0.7
Total	161	100.2

Mean LD activity, 470 units ± 130 units.

Results. 1. *Lactic dehydrogenase activity in blood* of human adults. The venous serum LD activity in 161 normal human adults varied from 260 to 850 units with a mean activity of 470 per m + 130 units (Table I). The LD activity found in whole venous blood hemolysates from normal human adults ranged from 16,000 to 67,000 units with a mean value of 34,000 ± 12,000 units per ml. The whole blood hemolysates were noted to be on the average approximately 100 times

TABLE II. Lactic Dehydrogenase Activity of Heparinized Plasma, Serum, and Whole Blood Hemolysate of the Same Venous Blood Samples.

Hematocrit (mm)	Whole blood hemolysate (units/ml)	Plasma (units/ml)	Serum (units/ml)
	×1000		
42	22	370	380
43	26	370	380
45	40	800	750
50	33	300	400
50	24	500	420
50	31	330	290
51	16	370	390
52	30	480	430
53	27	830	340
54	30	500	360

TABLE III. Estimated Lactic Dehydrogenase Activity of Tissues of the Dog.

Tissue	Units/g wet tissue
	(×1000)
Kidney	640
Skeletal muscle	600
Liver	390
Heart	240
Pancreas	150
Spleen	140
Brain	130
Lung	25

more active than the serum of the corresponding venous blood samples, but no consistent relationship was noted between serum and corresponding whole blood hemolysate LD activities, and none between whole blood hemolysate activity and hematocrit (Table II). The LD activity of plasma obtained from heparinized venous blood samples was not significantly different from the activity of serum from a corresponding venous blood sample, oxalated plasma was considerably less active suggesting oxalate inhibition and/or interference. In no instance was LD activity absent in the sera of normal human adults tested or in any of the sera of hospitalized patients with various disease states. The fasting did not appear to significantly influence the LD activity, and no consistent alteration in LD activity was observed during glucose tolerance studies in normal or in diabetic individuals. Day to day serum LD activities in the same individual varied 30% (within the normal range). Duplicate determinations on the same serum samples varied by 10% or less. LD activity was not connected with sex.

A sampling was made of patients hospitalized at Memorial Center with various diseases and serum LD activity determined in each instance. Activities within the normal range were obtained on sera from a limited selection of patients with infectious, degenerative, neoplastic and other disease states. High activities were observed in patients with myocardial infarction, diabetic acidosis, acute stem cell leukemia, chronic myelogenous leukemia and hepatitis.

2. *Lactic dehydrogenase activity* in the *tissues* and *blood* of experimental animal. LD activity was demonstrable in all the serum samples obtained from several experimental animals. LD activity in the venous sera of the experimental animals studied varied from 380 units per ml in the rabbit to 3100 units per ml in the rat with progressively diminishing values in between these 2 extremes in the dog, hamster, mouse and guinea pig. Using aliquot samples of macerated homogenized dog tissues obtained immediately after the death of the animal, the LD activity of various tissues was estimated and are summarized in Table III.

Following the intravenous administration of 0.13 ml of lactic dehydrogenase† diluted with saline to 5.0 ml (equivalent to approximately 240,000 units of LD activity or about one gram of dog heart muscle homogenate) to a 12.5 kilo dog, the serum was sampled at periodic intervals and examined for LD activity as indicated in Fig. 1.

Fig. 2 describes the serum LD alterations following experimental myocardial infarction, produced by the closed chest technic(5). (The detailed observations of serum and cardiac tissue LD activity elevations seen in experimental graded myocardial infarction and in clinical myocardial infarction will be the subject of a subsequent report.) As seen in the case of glutamic oxaloacetic transaminase, serum LD rises during the course of myocardial infarction. Similar alterations in serum LD activity have been observed in clinical myocardial infarction (Fig. 3).

Discussion. Lactic dehydrogenase activity

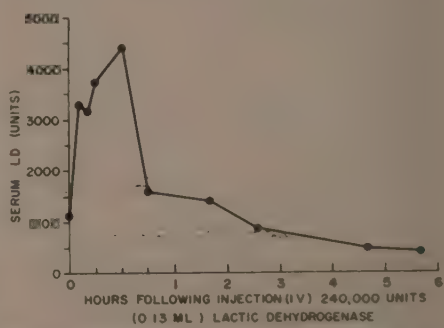

FIG. 1. Serum lactic dehydrogenase activity in a 12.5 kilo dog following the intravenous injection of approximately 240,000 units of lactic dehydrogenase.

† Obtained from Nutritional Biochemical Corp., Cleveland, O.

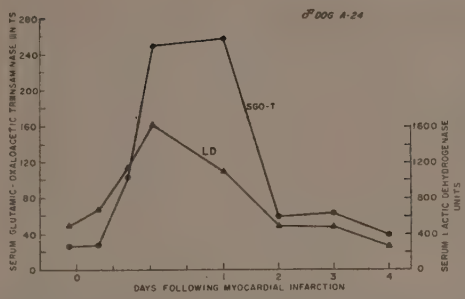

FIG. 2. Serum lactic dehydrogenase alterations during the course of closed chest experimental myocardial infarction in the dog. Comparison with serial changes in serum glutamic oxaloacetic transaminase is shown.

is present in all human sera and in all whole blood hemolysates examined. The previously reported serum glutamic oxaloacetic transaminase activity alterations in the course of myocardial infarction appear to be mimicked by similar changes in LD activity. That the latter changes are parallel but not related to serum glutamic oxaloacetic transaminase activity alterations is indicated by a comparison of serum glutamic oxaloacetic transaminase and LD activity observed during the course of clinical hepatitis. The LD and serum glutamic-oxaloacetic transaminase activity alterations following myocardial infarction are comparable in direction and degree but probably independent of one another. The fact that elevation of both LD and serum glutamic oxaloacetic transaminase follow heart muscle injury suggests that these enzymes are liberated from the damaged muscle cells into the blood stream. If such is the case for these

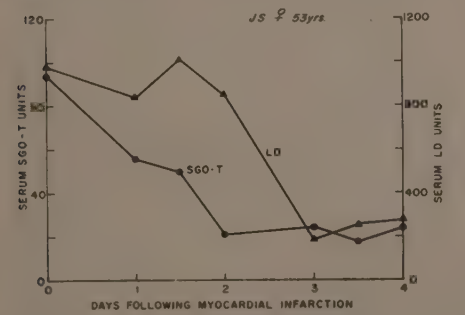

FIG. 3. Serum lactic dehydrogenase alterations during the course of clinical anterior myocardial infarction. Comparison with serial changes in serum glutamic oxaloacetic transaminase is shown.

two enzymes, there is every reason to expect that other cardiac tissue enzymes, when present in sufficient concentrations in the heart muscle, would also be released following damage to the heart muscle cells with a consequent change in the serum enzyme activity. This generalization is presently under investigation.

Summary and conclusions. 1. Lactic dehydrogenase activity is present in the venous serum of normal human adults. Normal activity ranges from 260 to 850 units per ml with a mean value of 470 ± 130 units per ml. 2. Venous whole blood hemolysates of normal adults have a lactic dehydrogenase activity varying between 16,000 to 67,000 units per ml with a mean value of 34,000 ± 12,000 units per ml. 3. Alterations in serum lactic dehydrogenase have been studied in a selected group of disease states. 4. Experimental and clinical myocardial infarction are associated with a rise in serum lactic dehydrogenase activity. 5. Lactic dehydrogenase like serum glutamic oxaloacetic transaminase rises in a characteristic fashion following myocardial infarction.

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1. LaDue, J. S., Wróblewski, F., and Karmen, A., *Science*, 1954, v120, 3117.

2. Karmen, A., Wróblewski, F., and LaDue, J. S., *J. Clin. Invest.*, 1955, v34, 126.

3. Agress, C. M., Jacobs, H. I., Glassner, H. F., Lederer, M. A., Clark, W. G., Wróblewski, F., Karmen, A., and LaDue, J. S., *Circulation*, 1955, v11, 711.

4. La Due, J. S., and Wróblewski, F., *ibid.*, 1955, v11, 871.

5. Nydick, I., Wróblewski, F., and LaDue, J. S., *Circulation*, 1955, v12, 161.

6. Meister, A., *J. Biol. Chem.*, 1950, v184, 117.

7. Straub, F. B., *Biochem. J.*, 1939, v33, 787.

8. ———, *ibid.*, 1940, v34, 483.

9. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, 1952, v194, 593.

10. Negelein, E., and Brömel, H., *Biochem. Z.*, 1939, v303, 231.

11. Kubowitz, F., and Ott, Paul, *ibid.*, 1943, v314, 94.

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Cytotoxic Action of Influenza Virus. Failure to Induce Acquired Resistance Phenomenon in Tissue Culture.* (21986)

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A number of substances modify the toxic action of influenza and related viruses in the intact animal. Sublethal doses of homologous or heterologous virus injected 24 hours prior to the challenge toxic inoculum prevent fever and lymphopenia in rabbits(1), convulsions in mice(2), and hemorrhagic encephalitis in chick embryos(3). Pretreatment of animals with *Cholera vibrio* filtrates also inhibits the pyrogenic(4) and neurotoxic(2) action of influenza viruses. Xerosin, a microbial product derived from *Achromobacter xerosis,* has the most profound inhibitory effect on the toxic pneumonitis(5) and encephalitis(6) caused by influenza and Newcastle disease viruses.

Henle, Girardi, and Henle(7) recently demonstrated that 4 strains of influenza virus produce a non-transmissible cytopathogenic effect in tissue cultures of a human epidermoid carcinoma (strain HeLa, Gey). Replication of infectious virus particles could not be demonstrated in this system but an increase in cellular non-infectious hemagglutinins occurred similar to the formation of "incomplete" virus in mouse brain that accompanies the neurotoxic action of influenza virus(8). This *in vitro* response of HeLa cells afforded a convenient method for determining whether biological inhibitors of influenza virus toxicity acted by interfering directly with host cell-virus interaction.

Materials. HeLa cell culture tubes were obtained from Microbiological Associates Inc. and incubated for an additional 24 hours at 35°-36°C before replacing the growth medium. The cells were then washed 3 times with 1 ml volumes of Hanks' balanced salt solution and the medium replaced with 0.8 ml of Scherer's maintenance solution and 0.2 ml of horse serum. After an additional 3 days of incubation a final change of medium was made to 10% horse serum-maintenance solution prior to inoculation of the cultures. Intact sheets of healthy cells usually persisted in control tubes for at least 3-4 days longer, the duration of the experiment. *Virus* preparations with high toxic activity were prepared by allantoic inoculation of 10-11-day-old chick embryos with a 10^{-6} dilution of seed material. After incubation for 48-72 hours at 35.5°C the allantoic fluids were harvested, pooled, tested for hemagglutinin (HA) content and stored at –20°C. It was not necessary to concentrate these preparations but since their cytotoxic action diminished on storage, fresh material was prepared frequently. To obtain virus preparations of lower toxicity, eggs were inoculated with undiluted seed(9) and incubated for 72 hours. Most experiments were carried out with an egg-passage line of the Lee strain of influenza B virus. Certain comparisons were made with the mouse-pathogenic WS strain and the mouse-unadapted A/England/1/51 strain of influenza A virus. *Cholera filtrate* was prepared from the 4Z strain of *V. cholerae* and used both as the crude filtrate and concentrated, purified material with receptor-destroying enzyme (RDE) activity (10) of 5,000 units/ml. *Xerosin* was kindly supplied by Dr. Vincent Groupé. It was

TABLE I. Cytotoxic Effect on HeLa Cell Cultures of Lee Virus Added to the Medium 24 Hr after Cholera Filtrate or Xerosin.

	Controls (NAF)	Lee virus
Distilled water	—	4, 4, 4, 4, 4
RDE, 10 units	0, 1, 0, 0, 0*	4, 4, 4, 4, 4
" , 100 "	0, 0, 0, 0, 0	4, 4, 4, 4, 4
Xerosin, 100 μg	0, 0, 0, 0, 0	2, 4, 4, 4, 4
" , 1000 " "	4, ±, 0, 1, 0	4, 4, 4, 4, 3

* 0, no cytopathogenic effect in 48 hr. 4, all cells destroyed. ± to 3, intermediate stages of cellular destruction.

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TABLE II. Influence of Subtoxic Virus, Cholera Filtrate and Xerosin Added to HeLa Cell Cultures 1 Hr before a 60% Cytotoxic Dose of Lee Virus.

	Controls (NAF)	Lee virus
Distilled water	0, 0, 0, 0	3, 2, 4, 2, ± 1, 3, 1, 2, 4
Subtoxic Lee virus	0, 0, 0, 0	3, 2, 4, 4, 4
RDE, 100 units	0, 0, 0, 0, 0	2, 3, 1, 1, 1
Xerosin, 1000 μg	0, 0, 0, 0	3, 3, 3, 4, 3

Read on scale of 0-4+ cytopathogenicity 72 hr after challenge.

dissolved in distilled water to the desired concentration and sterilized by autoclaving.

Results. Inoculation of HeLa cell cultures with 0.1 ml of undiluted "toxic" Lee allantoic fluid produced characteristic cytopathogenic effects. In 24 hours the cells were usually maintained as an intact sheet but islands of rounded cells with granular cytoplasm could be seen. By 48 hours after virus inoculation a considerable amount of destruction and clumping of cells was noted and by 72 hours most of the cells had dropped off the glass and could be seen floating in the medium. A 10-fold dilution of the Lee virus inoculum resulted in delayed and incomplete cytopathogenic effects and with a 1:100 dilution most of the cultures were unaffected. Allantoic fluid preparations of Lee virus made from undiluted seed, but possessing the same hemagglutinating activity as the "toxic" virus, were approximately one-tenth as potent.

Various concentrations of "subtoxic" virus, cholera filtrate and xerosin were incorporated into the maintenance medium, allowed to react with the cells, and the cultures challenged one or 24 hours later with 0.1 ml of toxic Lee virus. "Positive" virus controls consisted of tubes incubated in horse serum-maintenance solution with equivalent amounts of distilled water; "negative" controls, containing the same concentrations of test materials, were "challenged" with 0.1 ml of normal allantoic fluid (NAF). Distilled water, 200 AD of subtoxic virus, cholera filtrate and NAF had no effect on the microscopic appearance of the HeLa cells even after prolonged incubation. Minimal, sporadic cellular changes were observed in tubes containing 1 mg of xerosin, but these could be readily differentiated from typical virus pathogenicity. Ordinarily 5 or more tubes were used for each concentration of test material and "blind" readings made every day for 4 days after virus challenge. The extent of cellular damage was expressed on a scale of 0 to 4+. The numbers of test cultures are not always equal because it was necessary to discard an occasional contaminated tube.

Table I shows that the presence in the media of high concentrations of cholera filtrate and xerosin for 24 hours prior to Lee virus challenge did not inhibit cytotoxicity. In another experiment, a series of HeLa cultures were exposed to 200 AD of sub-toxic virus, 100 RDE units of cholera filtrate or 1 mg of xerosin and challenged one hour later with a preparation of Lee virus that produced only a 60% cytopathogenic effect in 72 hours. Under these conditions, it was again noted that these materials were ineffective as anticytotoxic agents (Table II). Lastly, in order to afford the most favorable conditions for demonstrating potential inhibition of cytotoxic action, a dose of toxic Lee virus was used that was capable of producing only 1+ cytopathogenicity in 72 hours. Cell cultures incubated for 24 hours with subtoxic Lee virus and xerosin were challenged with this minimally effective concentration of Lee virus. As shown in Table III, not only was there no inhibition of cellular damage, but pretreatment of cells with subtoxic virus or as little as 1 μg of xerosin enhanced the cytotoxic action of Lee virus in the 72-hour cultures. Even washing the cells free of xerosin and replacing the maintenance solution prior to virus inoculation rendered the cells more susceptible to the cytotoxic effects.

Many of these experiments were repeated with the WS and A/England/1/51 strains of influenza A virus. Although these viruses had more limited cytopathogenic action than the Lee strain, it was again noted that homologous or heterologous subtoxic virus, 100 RDE units of cholera filtrate (purified and crude) and 100-1000 μg of xerosin were incapable of preventing their *in vitro* cytotoxicity. In contrast, all of these materials effectively counteracted the neurotoxic action of the 3 strains of influenza virus. Mice injected intracerebrally with 200 AD of virus, 50 RDE units of cholera filtrate, or 30 μg of xerosin were highly re-

TABLE III. Apparent Enhancement of Minimally Cytotoxic Lee Virus by Subtoxic Virus and Xerosin.

	Control readings		Lee virus readings	
	72 hr	96 hr	72 hr	96 hr
Distilled water	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 1, 1, 1	4, 3, 3, 4, 3
Subtoxic Lee virus	0, 0, 0, 0, 0	0, 0, 0, 0, 1	3, 1, 1, 3, 2	4, 4, 2, 4, 4
Xerosin, 1 μg	0, 0, 0, 0	0, 0, 0, 0	4, 3, 4, 4, 4	4, 4, 4, 4, 4
" , 1 " (washed)	—	—	3, 3, 4, 4	4, 4, 4, 4

* Cultures incubated with 1 μg of xerosin for 24 hr and washed 3 times before virus challenge.

sistant to the convulsive and lethal effects of 5 LD_{50} of influenza virus administered 24 hours later.

Discussion. In the original studies(1) on acquired resistance of the intact host to virus toxins, the evidence seemed to support a local alteration in cellular susceptibility as the mechanism of action. This conclusion was based in part on the known capacity of virus and cholera filtrate to destroy the cell membrane receptors for influenza viruses(10). However, it was subsequently demonstrated that the degree of tolerance to the fever-producing(11,12) and neurotoxic(2) effects of influenza and mumps virus was not dependent on the enzymic action or receptor-gradient position of the protective virus. In addition, it is now quite clear that the antitoxic property of cholera filtrate is unrelated to receptor-destroying enzyme. Wagner(2) intimated that partially purified RDE was less effective in producing resistance to virus neurotoxin than crude cholera filtrate and French(11) proved that highly purified, concentrated RDE had no antipyrogenic activity. Finally, Groupé and coworkers(13) conclusively ruled out RDE as the antineurotoxic principle by demonstrating that autoclaved cholera filtrate, devoid of receptor-destroying activity, was almost as effective as unheated cholera filtrate. In spite of these studies, the likelihood persisted that resistance to virus neurotoxicity was a local cellular phenomenon since the protective effects of both subtoxic virus and cholera filtrate were manifested only by intracerebral inoculation(2).

Unlike influenza virus and cholera filtrate, xerosin does not affect cellular virus receptors(5) and induces some tolerance to toxic pneumonitis(5) and encephalitis(6) when administered parenterally subsequent to virus challenge. Nevertheless, it is most effective when injected intracerebrally 24 hours before influenza virus and the duration of tolerance is strikingly similar to that produced by subtoxic virus and cholera filtrate. For these reasons, Groupé and Hermann(6) postulated that xerosin might have two modes of action, one directly on host cell-virus interaction and the other indirectly on the host response. However, studies on xerosin inhibition of toxic pneumonitis led Ginsberg(14) to conclude that its influence was exerted purely by bolstering host resistance. He found that xerosin was effective by the subcutaneous route, and that it did not suppress the formation of "incomplete" virus nor prevent virus destruction of bronchiolar epithelium. Lastly, Ginsberg emphasized the nonspecific nature of xerosin-induced resistance by showing that it protected mice again non-viral, chemical pneumonitis.

The present studies demonstrated that the cytotoxic effects of influenza virus on HeLa cell cultures were not diminished by doses of subtoxic virus, cholera filtrate or xerosin in excess of those required to protect mice against viral neurotoxic action. The tissue culture methods assured direct exposure of susceptible cells to high concentrations of presumed antitoxic substances. If it can be assumed that the neurotoxic and cytopathogenic actions of influenza virus are related phenomena, it would appear that tolerance of the intact host is not due to local alteration in cellular susceptibility. It is apparent that the whole question of the mechanisms of acquired resistance to virus toxic action requires reexamination.

Summary. Subtoxic virus, cholera filtrate

and xerosin failed to protect HeLa cell cultures against the *in vitro* cytopathogenic effects of the Lee strain of influenza B virus and the WS and A/England/1/51 strains of influenza A virus. Smaller doses of these materials effectively rendered mice tolerant to the neurotoxic action of influenza viruses. The possible implications of these studies on the mechanisms by which the intact host acquires resistance to virus toxic action are discussed.

1. Bennett, I. L., Jr., Wagner, R. R., and LeQuire, V. S., *J. Exp. Med.*, 1949, v90, 335.

2. Wagner, R. R., *Brit. J. Exp. Path.*, 1952, v33, 157.

3. Burnet, F. M., and Fraser, K. B., *Aust. J. Exp Biol. M. Sc.*, 1952, v30, 447.

4. Wagner, R. R., and Bennett, I. L., Jr., *J. Exp. Med.*, 1950, v91, 135.

5. Groupé, V., Pugh, L. H., and Levine, A. S., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 710.

6. Groupé, V., and Hermann, E. C., Jr., *J. Immunol.*, 1955, v74, 249.

7. Henle, G., Girardi, A., and Henle, W., *J. Exp. Med.*, 1955, v101, 25.

8. Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 541.

9. McKee, A. P., *J. Immunol.*, 1951, v66, 151.

10. Burnet, F. M., and Stone, J. D., *Aust. J. Exp. Biol. M. Sc.*, 1947, v25, 227.

11. French, E. L., *ibid.*, 1952, v30, 479.

12. Wagner, R. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 612.

13. Groupé, V., Hermann, E. C., Jr., and Daugherty, R. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 636.

14. Ginsberg, H. S., *Fed. Proc.*, 1954, v13, 494.

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Effect of Epinephrine Upon Level of Plasma Amino Acids in the Eviscerate Rat. (21987)

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Some hormones affect the level of plasma amino acids in the eviscerate animal. The effect of epinephrine upon the level of plasma amino acids has not been studied in the eviscerate preparation although it has an effect in the intact animal(1). In the present study of eviscerate rats, doses of epinephrine which had striking effects upon glucose tolerance did not affect the level of plasma amino acids in either the presence or absence of insulin until large doses of epinephrine were administered with glucose and insulin for 18 hours.

Methods. Male rats of the Sprague-Dawley strain were fed Rockland rat diet. At a weight of 250 ± 5 g non-fasted rats were anesthetized with cyclopal sodium and functionally eviscerated. The intestinal tract was removed but the liver remained *in situ* after its arterial and portal vessels were tied. Continuous intravenous infusions of solutions of glucose with and without insulin and with and without epinephrine (Parke-Davis Adrenalin Chloride) were made by a continuous injection machine. The fluid was delivered into the saphenous vein of the right hind leg at the rate of 20 cc/24 hours/rat. Six rats were infused at a time so that the control and experimental animals were studied simultaneously. Temperature was constant at 26° ± 0.50C.

At the end of the infusion period the blood was drained from the abdominal aorta by cannula. Heparin was used as an anticoagulant. The level of plasma amino acids was determined by the method of Hamilton and Van Slyke(2) and that of blood glucose by the method of Miller and Van Slyke(3).

Results. The conditions of each experiment and the results are shown in Table I. In agreement with earlier reports(1) insulin suppresses the rate of rise of plasma amino acids following evisceration in the rat. During a 3-hour period epinephrine failed to affect the level of plasma amino acids in either the presence or absence of insulin although in the presence of insulin and a glucose load of 70 mg/100 g of rat per hour the

TABLE I. Level of Plasma Amino Acids in Functionally Eviscerate Rats. Averages* and standard errors.

Exp.	Condition	Glucose load, mg/100 g/rat/hr	Units insulin per 24 hr	Epinephrine, parts/million in 20 cc/24 hr	Hours	Concentration, mg % — Plasma amino acids — Epinephrine	Plasma amino acids — Control	Blood glucose — Epinephrine	Blood glucose — Control
1	Normal	0	0	0	0		5.46 ± .91		125 ± 4.6
2	Eviscerate	16	0	40	3	10.08 ± .39	9.48 ± .23	109 ± 4.7	179 ± 11.2
3	"	16	0	20	3	8.89 ± .24	9.12 ± .32	164 ± 8.55	160 ± 7.85
4	"	16	0	10	3	8.96 ± .37	9.44 ± .33	180 ± 9.89	167 ± 7.82
5	"	16	16	10	3	3.95 ± .30	3.98 ± .31	58 ± 3.48	48 ± 2.06
6	"	70	16	10	3	3.54 ± .16	3.77 ± .23	230 ± 13.33	79 ± 8.13
7	"	70	16	20	3	4.13 ± .12	4.11 ± .20	203 ± 16.7	86 ± 10.15
8	"	70	16	5	3	3.55 ± .09	3.87 ± .08	232 ± 8.79	73 ± 3.95
9	"	6	0	5	16	37.49 ± 1.34	35.16 ± 1.72	229 ± 27.93	193 ± 13.46
10	"	6	0	10	16	30.09 ± 2.38	30.3 ± 1.41	178 ± 17.09	229 ± 19.62
11	"	44	16	5	16	12.73 ± 1.05	11.67 ± .71	166 ± 24.74	145 ± 21.1
12	"	44	16	10	18	16.03 ± .69	12.88 ± 1.18	256 ± 35.3	137 ± 18.7
13	"	44	16	20	18	17.20 ± .73	11.94 ± .51	389 ± 30.9	118 ± 14.35

* Each average represents 12-15 rats.

addition of epinephrine caused a striking rise in the level of blood glucose (experiments 6, 7, and 8). During a 16-hour period epinephrine failed to affect the level of plasma amino acids in eviscerated rats given glucose without insulin. When insulin was given with an increased glucose load the addition of epinephrine in doses of 10 and 20 parts per million caused a significant increase in the level of plasma amino acids (experiments 12 and 13) during a period of 18 hours.

Discussion. The effects of epinephrine and insulin upon the glucose tolerance of the eviscerate rat are in agreement with published data(4). Although large doses of epinephrine given with glucose and insulin for 18 hours have a positive effect upon the level of plasma amino acids in the eviscerate rat there is no clear relationship between the changes in the level of blood glucose and the changes in the level of plasma amino acids. The possible physiological significance of this extra-hepatic effect of large doses of epinephrine upon the level of plasma amino acids is not known. Adrenodemedullated rats do not differ from normal rats with respect to the release of amino acids after evisceration(5).

Summary. Functionally eviscerated rats were given continuous intravenous injections of glucose with and without insulin, with and without epinephrine for periods of 3, 16, and 18 hours. Large doses of epinephrine given with glucose and insulin for 18 hours caused a significant rise in the level of plasma amino acids.

1. Russell, J. A., *Fed. Proc.*, 1955, v14, 696.

2. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, 1943, v150, 231.

3. Miller, B. F., and Van Slyke, D. D., *ibid.*, 1936, v114, 583.

4. Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol.*, 1949, v156, 361.

5. Frame, E. G., and Russell, J. A., *Endocrinol.*, 1946, v39, 420.

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Microbiological Studies on Growth Factor for *L. bifidus* var. *pennsylvanicus*.* (21988)

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For *L. bifidus* var. *pennsylvanicus*(1) human milk furnishes a specific and essential growth factor (factors), named in short the "bifidus factor" (BF). In addition, this particular variant of *L. bifidus* is able to utilize only preformed pantethine but not pantothenic acid, even when the latter is offered in great excess. The growth of *L. bifidus* var. *pennsylvanicus* is further enhanced by a supplementary factor, present in human milk, cow's milk, insulin and pancreatic extract. For some mutants (or variants) this supplementary factor may act as an essential nutrient. The chemical characteristics and distribution of the supplementary factor appear to be closely related to those of strepogenin. The supplementary factor is different from the specific "bifidus factor"(2). Chemically the bifidus factor as it occurs in human milk belongs in the group of N-containing carbohydrates. In human milk the presence of a great variety of oligo- and polysaccharides has been demonstrated(3). Their total quantity in fresh human milk may be estimated to be around 0.4% which is by no means a negligible amount with respect to total solids. The bifidus factor in human milk is present in both low molecular dialyzable and high molecular, non-dialyzable form(4). The bifidus factor may be adsorbed from deproteinized human milk on charcoal and eluted by acetic acid(3). Such milk eluates contain also dialyzable and undialyzable fractions(4). The carbohydrates of human milk as obtained in the acetic acid eluates from charcoal adsorbates are composed of glucose, galactose, fucose and N-acetyl-D-glucosamine, in various combinations(3). Kuhn and his associates have found in human milk one non-N-containing triose (fucosido-lactose) and a group of N-containing oligosaccharides, consisting of a tetraose, 2 pentaoses and one hexaose. They have also determined the composition and exact structure of several of these oligosaccharides and of some of their breakdown products, such as of 2 bioses and 2 trioses obtained from the tetraose(5-10). In addition to the neutral oligo- and polysaccharides, human milk also contains acidic oligo- and polysaccharides, which occur again in dialyzable, low molecular and non-dialyzable, high molecular form. The acidic constituent of these saccharides has been isolated, chemically identified and tentatively named gynaminic acid(11). It is probably N-acetylneuraminic acid(11-13). Upon hydrolysis the oligo- and polysaccharides containing gynaminic acid yielded glucose, galactose, fucose and N-acetyl-D-glucosamine, in addition to gynaminic acid(11). The common link in all these N-containing oligo- and polysaccharides is the presence of N-acetyl-D-glucosamine. This is equally characteristic for all microbiologically active compounds obtained from other natural sources(14). As a matter of fact, N-acetyl-D-glucosamine, as well as N-acetyl-D-galactosamine and even inorganic ammonium salts have been found, in high concentration, active, probably as precursors of the bifidus factor(14). All chemically well defined oligosaccharides of human milk,† various disaccharides obtained from different sources and also through enzymatic or chemical synthesis, and finally a series of synthetic derivatives of N-acetyl-D-glucosamine have been tested microbiologically.

The present report summarizes the results of these studies.

Methods. The microbiological assays were carried out as previously described(1), with the semisynthetic basal medium(15,16) containing an enzymatic digest of casein ("NZ case peptone," Sheffield). The supplements were used in varying concentration and were

* Supported by a grant of Wyeth Laboratories, Division of American Home Products Corp.

† Most compounds from human milk, as well as from other sources, and a large number of synthetic compounds were kindly furnished by Professor Richard Kuhn, Heidelberg.

TABLE I. Saccharides Isolated from Human Milk.

No.	Rf (lactose)	Name	Composition	Ref.	Microbiological activity, mg/unit Autoclaved	Sterile
V	1.0	Lactose			Inactive	Inactive
IV	.73	N-free triose	Fucose Glucose>galactose	10		Inactive
IIIc	.36	Lacto-N-tetraose	Glucose>galactose>N-acetyl-D-glucosamine-galactose	3, 5, 6	2.4	1.7
	1.49	Lacto-N-biose I	N-acetyl-D-glucosamine-galactose (3-0-β-D-galactopyranosyl-N-acetyl-glucosamine)	6, 8	Inactive	1.5
	1.12	Lacto-N-biose II	Galactose>N-acetyl-D-glucosamine	6, 7	.6	.2
	.51	Lacto-N-triose I	Galactose>N-acetyl-D-glucosamine-galactose	6, 7	1.2	.75
	.70	Lacto-N-triose II	Glucose>galactose>N-acetyl-D-glucosamine	6, 7	.2	.2
IIIb	.27	Lacto-N-fuco-pentaose II	Fucose Glucose>galactose>N-acetyl-D-glucosamine>galactose	9	.75	.26
IIIa	.19	Lacto-N-fuco-pentaose I	Fucose Glucose>galactose>N-acetyl-D-glucosamine>galactose	9	.3	.25
IIc[a]	.11	Lacto-N-difuco-hexaose	Fucose Fucose Glucose>galactose>N-acetyl-D-glucosamine>galactose	9	1.1	.38
	Rf (gynaminic acid)					
II	.58	Acid saccharide II	Gynaminic acid; galactose; glucose; N-acetyl-D-glucosamine; fucose	11	.4	.4
I	.15	*Idem* I	Similar to II but higher in mol. wt	11	.15	.15

[a] *Cf.* Ref. 3.

TABLE II. Disaccharides of N-acetyl-D-glucosamine from Other Sources than Human Milk.

Name of compound	Origin	Ref.	Microbiological activity, mg/unit Auto-claved	Sterile
4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine	From hog stomach mucin	17	.08–.1	.06
	Enzymatically synthesized	18, 19, 20	.08–.1	.06
	From meconium	21	.08–.1	.06
	Chemically synthesized	22	.08–.1	.06
3-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine	Enzymatically synthesized	23	Inactive	1.5
6-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine	Chemically synthesized	24	1.7	.95
	Enzymatically synthesized	18, 20	8	.90
6-0-β-D-(2′-deoxy-2′-acetamino)-glucopyranosyl-D-galactose	Chemically synthesized	25	.17	.17
6-0-β-D-(2′-deoxy-2′-acetamino)-glucopyranosyl-D-glucose	*Idem*	25	.10	.07
N-N′-diacetyl-chitobiose	From chitin	26	.8–1.8	1.2–2.2

either autoclaved with the medium or were added to it sterile (Seitz-filtered). The microbiological activity was expressed in mg per unit of the compound tested, related to the reference standard of skimmed human milk (1).

Results. Table I summarizes the results of the microbiological assays carried out with chemically well characterized compounds isolated from human milk. The Rf-values for the neutral oligosaccharides are based on that of lactose as unity(3), those for the acid saccharides are related to that of gynaminic acid (11). As expected, lactose and the N-free triaose (fucosido-lactose) were microbiologically inactive. Great variation of activity was found for all N-containing oligosaccharides tested. Among the neutral oligosaccharides only lacto-N-triose II and lacto-N-fuco-pentaose I have shown high activity, regardless whether they were autoclaved with the medium or added to it sterile. High activity was also found for the acid saccharides, both in autoclaved and in sterile form. A few other compounds were highly active when used Seitz-filtered, but showed greatly reduced activity after autoclaving with the medium. It deserves special emphasis that 3-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine, which is widely distributed in the N-containing oligosaccharides of human milk(6,8) exhibited no activity when autoclaved with the medium, and only very slight activity as sterile supplement. In contrast (Table II), 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine showed the highest microbiological activity of all the naturally occurring derivatives of N-acetyl-D-glucosamine. This disaccharide, originally isolated from hog mucin (17), is present in human milk in only low concentration, especially when compared with 3-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine. It has been obtained also from meconium(21), and through enzymatic(18,19) and chemical(22) synthesis, always with identical microbiological activity. In Table II several other disaccharides of N-acetyl-D-glucosamine are listed, some of them showing good microbiological potency.

In addition to the N-containing oligosaccharides, several synthetic derivatives of glucosamine were tested for microbiological activity (Table III). The α-anomers of the alkyl glyosides were found to be inactive. Unexpectedly very high activity was encountered with the β-alkyl compounds, in particular with the ethyl- and n-propyl-homologues. Even the benzyl and phenyl derivatives were strongly active. The activity of methyl-N-acetyl-β-D-glucosaminide was greatly enhanced(28) by the addition of the α-anomer and brought to the level of the pure ethyl- and n-propyl-compounds. On the basis of molecular weight the activity of these alkyl-derivatives of N-acetyl-D-glucosamine was comparable with that of 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine. In this connection it may be added that in the course of

TABLE III. Synthetic Derivatives of N-Acetyl-D-Glucosamine.

Name of compound	Ref.	Microbiological activity, mg/unit Autoclaved	Sterile
Methyl-N-acetyl-α-D-g*	27,28,29	Inact.	Inact.
Ethyl-N-acetyl-α-D-g	29	"	"
n-Propyl-N-acetyl-α-D-g	29	"	"
Benzyl-N-acetyl-α-D-g	†	1.5	—
Phenyl-N-acetyl-α-D-g	‡	Inact.	—
Methyl-N-acetyl-β-D-g	28,29,30,33	.16-.2	—
Ethyl-N-acetyl-β-D-g	29,30	.04-.06	.05
n-Propyl-N-acetyl-β-D-g	29,30	.04-.06	.06
Isopropyl-N-acetyl-β-D-g	†	.100	.09
n-Butyl-N-acetyl-β-D-g	30†	.065	
Benzyl-N-acetyl-β-D-g	30†	.095	
Phenyl-N-acetyl-β-D-g	†	.05-.07	.05
N-formyl-D-g	†	2.5	
Methyl-N-formyl-β-D-g	33†	2.5	
Aniline-N-acetyl-D-g	†	Inact.	
p-Anisidine-N-acetyl-D-g	†	"	
p-Aminobenzoic acid ethyl ester N-acetyl-D-g	†	.7	

* g = glucosamine.
† Kindly furnished by Dr. R. Kuhn, Heidelberg.
‡ " " " Dr. S. Roseman, Ann Arbor.

the last 2 years the sensitivity of the strain of *L. bifidus* var. *pennsylvanicus* to pure N-acetyl-D-glucosamine autoclaved with the medium has shown a distinct, gradual deterioration from 2.3 mg per unit to 4.5-7.0 mg. No such reduction in sensitivity was noticed for the bifidus factor, as it is present in human milk, the active disaccharides, and the alkyl-derivatives of N-acetyl-D-glucosamine.

Discussion. In reviewing the results of the microbiological tests reported it is apparent that the various oligo- and polysaccharides and other derivatives of glucosamine have shown considerable variation in their activity as bifidus factor. The most potent compound of natural origin was the disaccharide 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine. However, considering the relatively low concentration of this compound in human milk (5,6), it is difficult to assign to it the role of *the* specific bifidus factor. Further, the specificity of this disaccharide as bifidus factor is at variance with the fact that alkyl-glycosides of N-acetyl-D-glucosamine, especially the ethyl- and n-propyl derivatives show approximately the same high activity. Chemically the alkyl-glycosides and the disaccharide are not strictly comparable.

It is of additional interest that in previous studies crude eluates from charcoal adsorbates of skimmed human milk or fractions obtained by alcohol precipitation of deproteinized milk (3,31) have often been found to have very high microbiological activity (about 250 γ per unit), comparable to the activity of above named simple derivatives of N-acetyl-D-glucosamine. Purified blood group substances with an activity of ca. 150 γ per unit were obtained from ovarian cyst fluid (32).

In the light of all these microbiological findings it is not possible yet to identify the bifidus factor with *one* specific compound.

Summary. 1. The results of microbiological assays on a large number of oligosaccharides obtained from human milk and on simple derivatives (disaccharides or simple synthetic compounds) of N-acetyl-D-glucosamine are reported. The high microbiological activity of 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine and of ethyl- and n-propyl-N-acetyl-β-D-glucosamine has not been exceeded by any other compound tested. 2. The "true" specific bifidus factor has either still to be found or several related compounds may act as precursors or in synergistic combination.

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1. György, P., Norris, R. F., and Rose, C. S, *Arch. Biochem. and Biophys.*, 1954, v48, 193.

2. György, P., and Rose, C. S., *J. Bact.*, 1955, v69, 483.

3. Gauhe, A., György, P., Hoover, J. R. E., Kuhn, R., Rose, C. S., Ruelius, H. W., and Zilliken, F., *Arch. Biochem. and Biophys.*, 1954, v48, 214.

4. György, P., Hoover, J. R. E., Kuhn, R., and Rose, C. S., *ibid.*, 1954, v48, 209.

5. Kuhn, R., Gauhe, A., and Baer, H. H., *Berichte*, 1953, v86, 827.

6. ———, *ibid.*, 1954, v87, 289.

7. ———, *ibid.*, 1954, v87, 1187.

8. Kuhn, R., Baer, H. H., and Gauhe, A., *ibid*, 1954, v87, 1553.

9. Kuhn, R., *Angew. Chemie.*, 1955, v67, 184.

10. Kuhn, R., Baer, H. H., Gauhe, A., *Ber.*, 1955, v88, 1135.

11. Zilliken, F., Braun, G. A., and György, P., *Arch. Biochem. and Biophys.*, 1955, v54, 564.

12. Hoover, J. R. E., Braun, G. A., and György, P., *ibid.*, 1953, v47, 216.

13. Klenk, E., and Faillard, H., *Z. physiol. Chem.*,

1954, v298, 230.

14. György, P., Kuhn, R., Rose, C. S., and Zilliken, F., *Arch. Biochem. and Biophys.*, 1954, v48, 202.

15. Norris, R. F., Flanders, T., Tomarelli, R. M., and György, P., *J. Bacteriol.*, 1950, v60, 681.

16. Hassinen, J. B., Durbin, G. T., Tomarelli, R. M., and Bernhart, F. W., *ibid.*, 1951, v62, 771.

17. Tomarelli, R. M., Hassinen, J. B., Eckhardt, E. R., Clark, R. H., and Bernhart, F. W., *Arch. Biochem. and Biophys.*, 1954, v48, 225.

18. Zilliken, F., Smith, P. N., Rose, C. S., and György, P., *J. Biol. Chem.*, 1954, v208, 299.

19. Zilliken, F., Smith, P. N., Tomarelli, R. M., and György, P., *Arch. Biochem. and Biophys.*, 1955, v54, 398.

20. Zilliken, F., Smith, P. N., Rose, C. S., and György, P., *J. Biol. Chem.*, in press.

21. Kuhn, R., and Kirschenbohr, W., *Ber.*, 1954, v87, 560.

22. ———, *ibid.*, 1954, v87, 1547.

23. Alessandrini, A., Schmidt, E., Zilliken, F., and György, P., unpublished data.

24. Kuhn, R., and Baer, H. H., unpublished data.

25. Kuhn, R., and Kirschenbohr, W., *Ber.*, 1954, v87, 384.

26. Zilliken, F., Braun, G. A., and György, P., *J. Am. Chem. Soc.*, 1955, v77, 1296.

27. Kuhn, R., Zilliken, F., and Gauhe, A., *Ber*, 1953, v86, 466.

28. Rose, C. S., Kuhn, R., Zilliken, F., and György, P., *Arch. Biochem. and Biophys.*, 1954, v49, 123.

29. Zilliken, F., Rose, C. S., Braun, G. A., and György, P., *ibid.*, 1955, v54, 392.

30. Kuhn, R., and Kirschenbohr, W., *Ber.*, 1953, v86, 1331.

31. Brown, G. A., Zilliken, F., and György, P., unpublished data.

32. Springer, G. F., Rose, C. S., and György, P., *J. Lab. and Clin. Med.*, 1954, v43, 532.

33. Kuhn, R., and Baer, H. H., *Ber.*, 1953, v86, 724.

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Effects of β-Sitosterol and Ferric Chloride On Accumulation of Cholesterol in Mouse Liver.* (21989)

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Since repeated experimental evidence has associated high cholesterol levels with atherosclerosis, various attempts have been made to lower blood and organ cholesterol content. The effects of several substances on cholesterol absorption have been studied using various experimental animals. Soybean sterols, consisting mainly of β-sitosterol, have been shown to decrease the accumulation of dietary cholesterol(1-5). Studies on dihydrocholesterol treated animals have shown that this substance is also effective in reducing cholesterol absorption(6-8). Moreover, it has been found that, in the cockerel, precipitation of bile acids by ferric chloride results in a reduction of blood cholesterol(9,10). On the other hand, in the case of the mouse and rat, evidence has been produced that soybean sterols fail to lower organ and blood cholesterol(11,12).

The object of this experiment is to study: first, the effectiveness of β-sitosterol in prevention of liver cholesterol accumulation; and second, the effectiveness of ferric chloride in a mammalian species.

Methods. Exp. I. Dietary β-sitosterol. Webster strain female albino mice weighing 15 to 20 g were used. Throughout the experiment all mice were fed *ad lib.* a basal fat-free, cholesterol-free diet, described in a previous paper(7), supplemented by adequate amounts of vit. A and D. After a 2-week preliminary period on this basal diet, the mice were divided into 7 groups. The controls, Group I, were continued on the basal diet. For the other 6 groups the basal diet was supplemented with 1% cholesterol. Cholic acid was also added to the various diets as follows: Groups II and V received 0.25%; Groups III and VI, 0.5%; and Groups IV and VII, 1%. In addition, diets for Groups V, VI, and VII contained

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TABLE I. Effect of β-Sitosterol and Cholic Acid on Total Mouse Liver Cholesterol.

Group and diet	No. of mice	Additions to basal fat-free diet (%)			Total cholesterol in liver (mg)	
		Cholesterol	Cholic acid	β-sitosterol	Per organ†	Per g body wt‡
I	12	—	—	—	10.6 ± 2.1*	.53 ± .11*
II	10	1	.25	—	36.1 ± 6.3	1.88 ± .28
III	9	1	.5	—	47.1 ± 9.1	2.40 ± .52
IV	10	1	1.0	—	48.0 ±13.3	2.20 ± .60
V	10	1	.25	2.5	30.0 ± 6.1	1.37 ± .23
VI	11	1	.5	"	24.9 ± 6.4	1.27 ± .33
VII	9	1	1.0	"	34.2 ± 6.8	2.04 ± .41

* Stand. dev.

† Significance of the difference in results of the following groups: (a) II & V, $P \cong 0.04$; (b) III & VI, $P < 0.01$; (c) IV & VII, $P < 0.01$.

‡ Significance of the difference in results of the following groups: (a) II & V, $P < 0.01$; (b) III & VI, $P < 0.01$; (c) IV & VII, $P \cong 0.5$.

2.5% β-sitosterol. All diets were fed *ad lib.* for a 3-week experimental period, during which food consumption for the 7 groups varied from 2.9 g to 3.4 g per mouse per day. After the experimental feeding, the mice were sacrificed, and the livers removed, weighed, and quick frozen. Total liver cholesterol was assayed by a procedure similar to the one used in our previous study(7).

Exp. II. Dietary ferric chloride. National Institute of Health strain female albino mice weighing 15 to 20 g were used. As in Exp. I, all animals were fed *ad lib.* a basal fat-free, cholesterol-free diet for an initial 2-week period. The mice were then divided into 5 groups. For all groups, the basal diet was supplemented with 1% cholesterol and 0.5% cholic acid. In addition iron, as ferric chloride, was added to the diets as follows: Group I received no iron; Group II received 0.05%; Group III, 0.5%; Group IV, 1%; and Group V, 2%. After being fed *ad lib.* on these experimental diets for 3 weeks, the mice were sacrificed, and the livers quickly removed, weighed and frozen. Total cholesterol determinations were made as in Exp. I.

Results. Table I presents the data on the effect of β-sitosterol and cholic acid on the accumulation of mouse liver cholesterol. As can be seen, increasing the dietary cholic acid increased the concentration of liver cholesterol up to a maximum value attained in Group III. Comparison of Groups II and V, III and VI, and IV and VII shows the effectiveness of β-sitosterol in preventing liver cholesterol build-up. It is to be noted that β-sitosterol is most effective in the presence of 0.5% cholic acid. However, in none of these groups did total cholesterol concentration approach the control values.

Results of simultaneous feeding of cholesterol and ferric chloride are shown in Table II. The data indicate that ferric chloride failed to decrease cholesterol accumulation. In fact, increasing the concentration of ferric chloride seemed to increase the cholesterol level. Even though comparison of Groups I and II may suggest a slight decrease, the significance of this difference is doubtful in view of the large standard deviation.

Discussion. From the data in Table I, it can be seen that β-sitosterol is effective in decreasing liver cholesterol accumulation under certain conditions. However, comparison of the present results with those obtained in an identical experiment(7) with dihydrocholesterol, shows that, in the presence of equal amounts of cholic acid, dihydrocholesterol is more effective than β-sitosterol.

Data on the ferric chloride experiment indicate that this compound does not reduce dietary cholesterol accumulation in liver. This finding does not coincide with that of Siperstein *et al.*(9), who postulated that ferric chloride binds the bile acids in the intestinal tract, thereby suppressing cholesterol absorption. However, it must be noted that two major differences exist between their experiment and the present one: First, Siperstein *et al.* experimented on cockerels, while in our experiment, mice were used; and second, they studied blood cholesterol levels, while our data

TABLE II. Effect of Ferric Chloride on Total Mouse Liver Cholesterol.

Group and diet	No. of mice	Additions to basal fat-free diet (%)			Total cholesterol in liver (mg)	
		Cholesterol	Cholic acid	Iron as $FeCl_3$	Per organ	Per g body wt
I	7	1	.5	—	56.3 ± 11.7*	2.81 ± .65*
II	8	1	.5	.05	43.9 ± 10.4	2.22 ± .38
III	7	1	.5	.5	53.5 ± 11.5	2.54 ± .46
IV	7	1	.5	1.0	68.6 ± 17.4	3.36 ± .65
V	7	1	.5	2.0	67.6 ± 7.6	3.76 ± .50

* Stand. dev.

are measurements of liver cholesterol concentrations. The differences found in these experiments may be explained by the hypothesis that ferric ions have a toxic action on liver cells, which prevents the normal distribution of cholesterol between the blood stream and the liver. In agreement with Siperstein *et al.*, we found that ferric chloride, fed in concentrations of 2% or more, proved to be highly toxic and, in some cases, fatal after a few days. The possible physiological after-effects of even small amounts of this compound cannot be overlooked.

Summary. 1. Increasing the cholic acid content of a diet rich in cholesterol, significantly increased total liver cholesterol. 2. The addition of β-sitosterol to diets containing cholic acid and cholesterol, decreased total liver cholesterol. 3. Addition of ferric chloride, in varying amounts, to diets rich in cholesterol and cholic acid, resulted in practically no change in total cholesterol content of mouse liver.

1. Peterson, D. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 143.

2. Peterson, D. W., Shneour, E., Peek, N., and Gaffey, H., *J. Nutrition,* 1953, v50, 191.

3. Pollak, O., *Circulation,* 1953, v7, 696.

4. Hernandez, H., Peterson, D. W., Chaikoff, I. L., and Dauben, W., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 498.

5. Alfin-Slater, R. B., Wells, A. F., Aftergood, L., Melnick, D., and Deuel, H. J., *Circulation Research,* 1954, v2, 471.

6. Rosenman, R. H., Byers, S. O., and Friedman, M., *ibid.,* 1954, v2, 45.

7. Beher, W. T., and Anthony, W. L., *J. Nutrition,* 1954, v52, 519.

8. Siperstein, M. D., Nichols, C. W., and Chaikoff, I. L., *Circulation,* 1953, v7, 37.

9. ———, *Science,* 1953, v117, 386.

10. Siperstein, M. D., Chaikoff, I. L., and Reinhardt, W., *J. Biol. Chem.,* 1952, v198, 111.

11. Schettler, G., *Klin. Wschr.,* 1948, v26, 566.

12. Rosenman, R. H., Byers, S. O., and Friedman, M., *Circulation Research,* 1954, v2, 160.

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Identification by Fluorescent Antibody of Developmental Forms of Psittacosis Virus in Tissue Culture.* (21990)

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Bedson(1) has described a developmental cycle for psittacosis elementary bodies in the cytoplasm of host cells. The cytologic studies (1-6) proved that the intermediate developmental forms such as initial bodies and plaques are regularly associated with the propagation of virus and are structural evidence of cellular infection. Morphologic observations have failed to establish whether the large structures are secondary reaction products or multiplying virus. Electron micrography has so far produced only equivocal evidence(7-9). The present study was undertaken with the hope that the immunocytochemical method for intracellular identification of antigen described by Coons and his coworkers(10) might determine the nature of the inclusion bodies.

Materials and methods. Virus: The psittacosis 6 BC strain was generously provided by Dr. Herbert Morgan, University of Rochester School of Medicine, and was maintained by yolk sac passage in embryonated eggs. A 10% yolk sac suspension with an LD_{50} titer of 10^{-8} was used. Albany strain mice were employed. Infectivity tests were performed in young adults (8-12 g). Pregnant mice 60-80 days old were the source of mouse embryos. Groups of 5 mice were inoculated intracerebrally with 0.03 ml of undiluted supernates of lightly centrifuged ground tissue suspension, then observed for 21 days. *Tissue culture*: Pregnant mice, approximately at the end of the period of gestation, were sacrificed by ether anesthesia. Embryonic livers were removed aseptically and minced with detachable knife blades into 1- x 2-mm pieces. Five pieces were planted on a 10- x 20-mm cover glass and 5 were planted on a 10- x 10-mm glass fiber mesh(11) placed in the flat bottom of a Leighton tube (Microbiological Associates, Bethesda, Md.). Approximately 0.1 ml of horse serum was added to the explants. Preparations were incubated at 40°C for 20 minutes, after which nutrient fluid(12) (beef amniotic fluid 85%, beef embryo extract 5%, horse serum 10%, 50 μg/ml of crystalline dihydrostreptomycin sulfate (Merck)) was added, 0.5 ml per tube. Cultures were incubated at 35°C for 5 days prior to use. *Fluorescent-antibody technic*: The indirect method of Weller and Coons(13), and Coons, Leduc, and Connolly(14) was applied. Fluorescein isocyanate was synthesized and conjugated to the pseudoglobulin fraction of antihuman gamma globulin horse serum‡ according to methods described by Coons and Kaplan(10) with minor alterations. For specific combination with intracellular antigen, human antipsittacosis serum (No. V54-2029) was used. The 50% unit titer was 1285 in a complement-fixation test against a phenolized 10% yolk sac antigen. A human serum (No. V54-1766) was the control. Nonspecific staining was eliminated by absorption of the conjugate with whole mouse embryo powder and subsequent dilution with buffered saline (sodium chloride solution, phosphate buffered to pH 7.0). Cover-glass preparations from inoculated and uninoculated groups were removed and treated as described by Weller and Coons(13). Stained cultures were examined for the presence of intracellular antigen under the fluorescence microscope. Its light source was a 1000-watt General Electric water-cooled mercury vapor lamp (type A-H6). The beam was condensed

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by a quartz lens (61.5 mm diameter, 59.5 mm focal length). Infrared and visible light was filtered by passing the beam through 25% $CuSO_4$ in a water-cooled cell in which a Corning glass filter (#5840, ½ standard thickness) was immersed. Slides were viewed through a dark-field microscope with a Wratten 2 B gelatin filter in the ocular. Photomicrographs were taken with a Zeiss Miflex camera and super ortho press film. Exposure for 400X magnification was 3 minutes. Films were tray-developed in DK-50 for 5 minutes at 20°C.

Results. Specificity of fluorescent antibody technic: In preliminary experiments, infected and uninfected explants were exposed for 20 minutes to 1:10 dilutions, respectively, of human antipsittacosis serum and of human serum which did not react in the complement-fixation test with psittacosis antigen. Cultures on cover glasses were washed for 10 minutes with buffered saline, then overlaid for 20 minutes with a 1:10 dilution of fluorescein conjugate. Cultures were again washed for 10 minutes and mounted as described by Weller and Coons(13). Brilliant yellow-green fluorescence was observed in the cytoplasm of infected cells treated with human antipsittacosis serum. Uninfected explants exposed to human antipsittacosis serum and infected explants treated with nonreacting human serum showed blue-gray autofluorescence.

Relation of inoculation dose to specific fluorescence and infective virus: Cultures with good outgrowth were divided into 3 groups inoculated respectively with 0.05 ml of 10^{-1}, 10^{-3}, and 10^{-5} dilutions in nutrient fluid of yolk sac suspension. At varying times (Table I) preparations of each group were treated in the following manner: 1. Cover-glass preparations were washed free of extracellular antigen in 3 changes of buffered saline. Exposure of infected cultures to human antipsittacosis serum was followed by staining with fluorescent antibody. Time factors for the staining procedure were substantially the same for all preparations. 2. Glass fiber patches carrying infected explants were removed from Leighton tubes and washed in 6 changes of buffered saline to minimize contamination by residual antigen, then ground

TABLE I. Comparison of Appearance of Specific Fluorescence and Presence of Infective Virus at Various Time Intervals following Inoculation of Different Doses of Virus.

Dil. of virus inoc.	Neg.* log of LD_{50} titer	Time after inoculation—specific fluorescence†												Time after inoculation—infective virus‡			Avg time until death (mice), days
		0 min.	10 min.	1 hr	3 hr	5 hr	8 hr	12 hr	16 hr	20 hr	24 hr	48 hr	72 hr	48 hr	72 hr	
10^{-1}	5.3	—	±	+	++	+++	+++	+++	+++	+++	+++	+++	+++	3/5	5/5	3– 6
		4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5			
10^{-3}	2.5	NT	NT	—	—	—	±	+	++	++	++	++	++	5–14		
				0/5	0/5	2/5	3/5	5/5	2/5	4/5	3/5	3/5	5/5			
10^{-5}	<1.0	—	—	—	—	—	—	—	—	—	—	±	±	20		
		NT	NT	NT	NT	NT	1/5	0/5	0/5	0/5	0/5	0/5	0/5			

* LD_{50} titer of ground tissue and nutrient fluid at 0 time.
† Grading of results explained in text.
‡ Presence of infective virus in washed tissue explants; numerator = No. of dead mice; denominator = No. of mice inoculated.
NT = Not tested.

Fig. 1

Fig. 2

Fig. 3

FIG. 1. Mouse embryo liver cells infected with 10^{-1} dose of virus; stained by fluorescent technic 72 hr after inoculation. Arrow points to plaque. 400×.

FIG. 2. Mouse embryo liver cells infected with 10^{-5} dose of virus; stained by fluorescent technic 72 hr after inoculation. 400×.

FIG. 3. Mouse embryo liver cells infected with 10^{-1} dose of virus; stained by Giemsa's method 72 hr after inoculation. Arrow points to plaque. 400×.

in mortars with alundum and 0.5 ml of nutrient fluid per 5 explants. After light angle centrifugation supernates were tested for infectivity in mice. In 10 different experiments, approximately 260 cover-glass preparations, carrying 5 explants each, were examined for presence of specific fluorescence at different intervals postinoculation. Results were graded from — to ++++, — indicating absence of specific fluorescence; ± a trace; + involvement of 5%; ++ 6-25%; +++ 26-75%; and ++++ the presence of specific fluorescence in more than 75% of the outgrowing cells. With the heaviest virus inoculum (10^{-1}), intensity of specific cytoplasmic fluorescence was moderate early and became marked 16 hours following inoculation and remained intense throughout the experiment (Fig. 1). With a medium (10^{-3}) dose of virus, two distinct features were noted: The appearance of specific fluorescence was delayed and its distribution was focal, involving 5-25% of outgrowing cells. The intensity was weak for the first 16 hours, and became moderate for the rest of the experiment. Negative results were obtained when the 10^{-5} dilution was used (Fig. 2).

Tests for the presence of virus in washed explants indicated that it was present in all groups inoculated with the 10^{-1} dilution. The average time to death decreased in the 10^{-1} and 10^{-3} groups as the experiment progressed, evidence of the multiplication of the virus.

Identification of antigen in developmental forms. In this series of experiments, the morphology of cultures stained with fluorescent antibody was compared with Giemsa§-stained cover-glass preparations. Diffuse fluorescence was observed in the early stages, 1, 3, and 5 hours postinoculation. Stippling, *i.e.*, the appearance of tiny fluorescent granules, distributed evenly throughout the cytoplasm, occurred as early as 8 hours following inoculation. It compared well with purple red staining elementary bodies and blue staining larger initial bodies in the cytoplasm of the cells stained by Giemsa's method. Concurrent with the blue staining vesicles and plaques at 12- and 16-hour intervals, larger fluorescing bodies were observed, sometimes eccentrically located in the cytoplasm. The size of the inclusion bodies in the Giemsa preparations continued to be much the same as in the fluorescent-antibody preparations throughout the growth cycle of the virus in cultures inoculated with the moderate (10^{-3}) and heavy (10^{-1}) doses (Fig. 3). Cultures in-

§ Giemsa stock solution, Merck and Company, Darmstadt, Germany.

oculated with the low (10^{-5}) dose, which showed no specific fluorescence, likewise developed no inclusion bodies detectable by Giemsa stain.

Discussion. By the immunocytochemical criterion described it was shown that antigen was regularly present within the developmental forms of psittacosis virus and that the inclusion bodies can be considered to be virus colonies rather than reaction products of infected cells. Bedson and Gostling(15) have suggested that multiplication of psittacosis virus is initiated as soon as elementary bodies are introduced into a susceptible tissue. This view is substantially supported by our *in vitro* observation that virus can be located within the cytoplasm of a few cells as early as one hour after inoculation. Whether the early intracellular appearance of antigen signifies the presence of infective virus has not been settled, since repeated washing of explants failed to remove traces of residual virus. In groups inoculated with a moderate dose, however, the early stages were characterized by lack of specific fluorescence and negative infectivity tests. With the appearance of intracellular fluorescence, infective virus was also demonstrated.

A large dose of virus infected 75% or more of outgrowing cells within the first 16 hours, whereas a smaller dose produced only a focal distribution of specific fluorescence, leaving 75% or more of outgrowing cells uninfected.

Weiss(5) has described intranuclear granules resembling elementary bodies 7 to 10 days after infection of experimental animals, and considered the possibility that the virus had invaded the nuclei. In our experiments specific fluorescence appeared within the cytoplasm alone during the 72-hour observation period. The various stages of cellular division were not correlated with the detection of specific fluorescence. No attempt was made to examine for extracellular forms of virus(5), since the search for intracellular antigen necessitated frequent washing of cultures to minimize residual virus. The fluorescent-antibody technic proved to be more sensitive than Giemsa's differential stain for detection of virus provided the inoculation dose was large. Giemsa staining failed to disclose with certainty intracellular inclusions at 1- and 3-hour intervals following inoculation, whereas the immunocytochemical stain detected intracellular antigen earlier and in greater quantity throughout the experiment. The difference may have been affected by the difference in method of fixation, formalin(16) being used for the Giemsa preparations, while air drying (1 hour at 37°C) followed by acetone was employed for the fluorescent-antibody studies. Antigen might be lost during the former treatment. Numerous observations of empty vesicles within the cytoplasm of Giemsa-stained cells suggested this might have been the case. A similar loss of antigen may have occurred during the studies of Kurotchkin *et al.*(7) who reported disintegration of large structures following treatment with ether for 5 minutes and reasoned that inclusion bodies represent secondary reaction products, perhaps of lipid nature, rather than virus colonies.

Summary. 1. Developmental forms of psittacosis virus have been studied in mouse embryo liver tissue cultures *in vitro* by the fluorescent-antibody technic. 2. Inoculation of a large dose of virus resulted in the intracellular appearance of antigen in most of the outgrowing cells within 16 hours. A moderate dose of virus resulted in the focal distribution of specific fluorescence. When a small dose of virus was inoculated, no specific fluorescence was detected. 3. The evidence indicates that virus antigen is present in the developmental forms within the cytoplasm of infected cells throughout the various stages that make up the growth cycle of psittacosis virus.

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1. Bedson, S. P., *Brit. J. Exp. Path.*, 1932, v13, 65.
2. Bedson, S. P., and Bland, J. O. W., *ibid.*, 1932, v13, 461.
3. Bedson, S. P., *ibid.*, 1933, v14, 267.
4. Bland, J. O. W., and Canti, R. G., *J. Path. and Bact.*, 1935, v40, 231.
5. Weiss, E., *J. Inf. Dis.*, 1949, v84, 125.
6. Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, v75, 323.
7. Kurotchkin, T. J., Libby, R. L., Gagnon, E.,

and Cox, H. R., *J. Immunol.*, 1947, v55, 283.

8. Rake, G., Rake, H., Hamre, D., and Groupé, V., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 489.

9. Gaylord, W. H., Jr., *J. Exp. Med.*, 1954, v100, 575.

10. Coons, A. H., and Kaplan, M. H., *ibid.*, 1950, v91, 1.

11. Warner, D., Hanawalt, C., and Bischoff, F., *J. Nat. Cancer Inst.*, 1949, v10, 67.

12. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.

13. Weller, T. H., and Coons, A. H., *ibid.*, 1954, v86, 789.

14. Coons, A. H., Leduc, E. H., and Connolly, J. M., *J. Exp. Med.*, 1955, v102, 49.

15. Bedson, S. P., and Gostling, J. V. T., *Brit. J. Exp. Path.*, 1954, v35, 299.

16. Herzberg, K., *Zentralbl. f. Bakt.*, 1954, v160, 481.

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Protection Against Tourniquet Shock Afforded by Parabiosis.* (21991)

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When rats are placed in parabiosis one of them may thereby be protected against otherwise fatal consequences. It has been shown for example, that survival of one is prolonged after total nephrectomy(1), a lethal dosage of x-rays(2,3) or bilateral adrenalectomy(4), when the co-twin is normal. Similarly the usually severe diabetes following either pancreatectomy(5) or toxic doses of alloxan(6) is either minimized or abolished by the presence of a normal partner. Recently we have reported that when one of a normal pair of parabiotic rats is subjected to transection of the spinal cord just caudad to C7, the operated rat develops erythremia, with increased hemoglobin, erythrocyte count and hematocrit, whereas the intact co-twin concurrently develops a rapidly fatal anemia(7). This study was interpreted to indicate that the capillary hypotension induced on one side of the capillary anastomosis by spinal transection led to a decrease in the amount of blood exchanged from operated to intact, while the amount traversing the vascular bed from intact to operated remained normal. These changes, typical of spontaneous parabiosis intoxication (8), suggest the latter to be due to a shock-like state in one of the partners.

The present study was undertaken to determine whether the disparity in blood transfer could also be induced by subjecting one of a pair to tourniquet shock, or whether the usually lethal effects of this intervention would be modified by the presence of a normal twin.

Materials and methods. The animals used in these studies were females of the Holtzman strain. Thirteen single animals weighing 100-139 g served as controls, and 10 pairs of parabionts, in union for 2 weeks and together weighing 200-280 g, constituted the parabiotic group. Parabiosis was performed essentially by the method of Bunster and Meyer(9). Tourniquets were applied to both hind limbs of the singles and to both hind limbs of one of the partners of each pair. The method was similar to that described by Rosenthal(10) and Stopack(11). A #8 elastic band was looped 5 times around a hollow tube of 16 mm diameter, each hind limb was then drawn through the tube and the elastic ligature slipped off so as to constrict the limb as close to the body as possible. The ligatures were removed 5 hours later. Rectal temperature, erythrocyte count, hematocrit and hemoglobin determinations were taken on all animals prior to ligation, and at 12, 36, and 48 hours after its removal, where survival permitted. Purina Laboratory Chow and tap water were available to the rats at all times.

Results. Of the 13 single rats to which the tourniquets were applied, 5 died within 12 hours of removal of the ligatures and all but one had succumbed by 48 hours thereafter.

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TABLE I. Effect of Tourniquet Shock on Single and Parabiotic Rats.

A.—Single rats

Time	No. rats	Rectal temp., °C	Erythrocytes, millions cu/mm	Hemoglobin, g/100 cc	Hematocrit
Before tourniquet	13	38.0 ± 1.4*	7.50 ± .30	14.9 ± .3	42 ± 1.2
12 hr after removal	8	29.4 ± 1.2	10.43 ± .36	20.1 ± .5	62 ± 1.0
36 " " "	2	28.8 ± .8	11.15 ± .83	21.5 ± .9	65 ± 3.1
48 " " "	1	38.4	9.80	15.3	51

B—Parabiotic pairs

Time	No. pairs	Rectal temp., °C		Erythrocytes, million cu/mm		Hemoglobin, g/100 cc		Hematocrit	
		Left†	Right	Left	Right	Left	Right	Left	Right
Before tourniquet	10	38.2 ± .3	38.2 ± .3	7.57 ± .26	8.15 ± .39	14.6 ± .3	15.0 ± .6	42 ± 1.2	44 ± 1.7
12 hr after‡	10	37.5 ± .2	37.7 ± .2	9.12 ± .39	8.05 ± .50	16.4 ± .2	14.6 ± .2	50 ± 1.1	43 ± 1.6
36 " "	10	38.0 ± .2	37.9 ± .2	7.09 ± .32	6.69 ± .59	13.0 ± .3	12.3 ± .9	37 ± 1.4	36 ± 2.3
48 " "	10	38.2 ± .1	38.2 ± .2	7.02 ± .42	6.66 ± .46	12.8 ± .4	12.6 ± .8	37 ± 1.6	38 ± 2.4

* ± S.E. of the mean. † Animal with tourniquet applied. ‡ After removal.

Rectal temperatures declined rapidly, and there occurred a marked hemoconcentration as evidenced by increase in the red-cell count, hemoglobin and hematocrit which was progressive until death. The results are given in Table IA.

The behavior of parabiotic rats was in marked contrast. Following removal of the tourniquets from the limbs of the one animal, there occurred a transient hemoconcentration at 12 hours, reflected in the hemogram, but no alteration in rectal temperature. However, at the 12-hour interval hemoconcentration was not nearly as marked in these as in single animals similarly treated, and whereas it was progressive in the latter it promptly reverted towards normal in the parabionts. Further, although the edema of the limbs was as marked in parabionts as in singles following ligature removal, and indeed in one the limbs amputated 4 days thereafter, none of them succumbed. The hemograms of the untreated partners indicated an anemic tendency. This was detectable at the 36-hour interval, was still present at 48 hours, and recovered slowly thereafter. Inasmuch as each of the pairs had tended to stabilize within 48 hours, no further blood determinations were made, although survival was followed for 2 weeks. The results are summarized in Table IB.

Discussion. Since the chief connection between the twins is vascular, and since the recovery from transient hemoconcentration in the injured rat was accompanied by hemodilution in the untreated partner, it seems reasonable to attribute the protective effect of parabiosis to the availability of the normal partner's blood to the injured twin. The magnitude of this interchange was sufficient to induce changes in the hemogram of the untreated animal in most cases although not to the same degree as has been true of untreated rats, attached to twins subjected to spinal transection, which invariably succumb. It would seem likely that the erythrocyte and fluid reserves available to the untreated twin are often sufficient to enable it to maintain an almost normal hemogram in the face of demands by the damaged twin. Only when the reserves are strained, as when a normal animal is attached to a twin with a spinal transection(7), to a dying or dead partner (12), or perhaps when one is attached to the hyperemic member of a pair with spontaneous parabiosis intoxication(12), is the resulting demand upon the vascular fluids of the normal partner sufficiently severe as to induce a fatal anemia. This is believed to be due to whole blood transfusion of one rat by its partner, with the latter undergoing hemodilution in an effort to sustain the declining blood volume as discussed elsewhere(13).

Summary. 1. Elastic ligatures applied to both hind limbs of young female rats for 5 hours produced shock and death in 12 of 13 single rats; a marked and progressive decrease

in body temperature and an increase in hemoconcentration invariably occurred in those which succumbed. 2. Tourniquets applied similarly to the limbs of one partner failed to produce a single death in 10 pairs of parabionts. A transient hemoconcentration was observed in the injured partner at 12 hours although rectal temperatures remained stable. Anemia occurred in the untreated member of many of the pairs, and is believed to reflect transfusion of the shocked rats, with compensatory hemodilution in an effort to sustain the blood volume.

1. Grollman, A., and Rule, C., *Am. J. Physiol.*, 1943, v138, 587.

2. Brecher, G., and Cronkite, E. P., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 292.

3. Finerty, J. C., Binhammer, R., and Schneider, M., *Texas Rep. Biol. and Med.*, 1952, v10, 496.

4. Mayeda, T., *Deutsche Z. f. Chir.*, 1921, v167, 295.

5. Fels, E., and Foglia, V. G., *Rev. Soc. argent. Biol.*, 1949, v25, 62.

6. Weitze, M., and Riggins, G. M., *Anat. Rec.*, 1954, v118, 857.

7. Hall, C. E., and Hall, O., *Fed. Proc.*, 1955, v14, 68.

8. Chute, R. N., and Sommers, S. C., *Blood*, 1952, v7, 1005.

9. Bunster, E., and Meyer, R. K., *Anat. Rec.*, 1933, v57, 339.

10. Rosenthal, S. M., *U. S. Pub. Health Rep.*, 1943, v58, 1429.

11. Stopack, J. H., *Am. J. Physiol.*, 1953, v175, 99.

12. Hall, C. E., and Hall, O., *Am. J. Physiol.*, (in press).

13. ———, *J. Exp. Med.* (in press).

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Inhibition of Lipid Synthesis by Alpha-Phenyl-N-Butyrate and Related Compounds. (21992)

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Cottet *et al.* have reported that feeding alpha - phenylbutyrate (phenylethylacetate) can lower serum cholesterol levels in the normal rat(1,2) and in patients with various forms of hypercholesterolemia(3). Since the drug did not prevent hypercholesterolemia in cholesterol-fed rats(1), it presumably does not act by inhibiting intestinal absorption; but the mechanism of action remains to be established. The present studies show that *in vitro* the drug strikingly inhibits synthesis of both cholesterol and fatty acids from sodium acetate-1-C^{14}.

Methods. Male Sprague-Dawley rats maintained on Purina rat chow to the time of sacrifice were decapitated and livers rapidly removed and chilled. 350-450 mg liver slices made with the Stadie-Riggs microtome were incubated with sodium acetate-1-C^{14} at 37°C in Krebs-Ringer phosphate buffer, pH 7.4, from which Ca^{++} and Mg^{++} were omitted. $C^{14}O_2$ was collected in a KOH center well and converted to $BaC^{14}O_3$ for assay of radioactivity. At the end of incubation the slices were drained of buffer and homogenized for 3 minutes in 50 ml 1:1 acetone-ethanol in a Waring blendor.* After centrifugation the supernatant was taken to dryness on a steam bath under nitrogen, and the dried residue saponified by autoclaving 4 hours at 15 pounds with 5 ml 3 N KOH. Then 5 ml absolute ethanol were added and the non-saponifiable lipid extracted with three 30-ml aliquots of n-heptane. This was back-washed with 1 N KOH and water. Aliquots of the concentrated heptane extract were dried on steel planchets and counted directly to determine *non-saponifiable lipid radioactivity*. In several experiments

* We are indebted to Dr. Joseph H. Bragdon for this simple procedure which he has found to give essentially complete extraction of liver lipids.

cholesterol radioactivity was measured by preparing and counting the digitonide. Results were essentially the same when obtained by direct counting of heptane extracts. On the basis of this correlation, the incorporation into *non-saponifiable lipid* fraction may be taken as a measure of cholesterol synthesis. The KOH phase, after extraction of cholesterol was acidified and the alcohol and volatile fatty acids removed by heating on a steam bath. Non-volatile fatty acids were extracted with three 30-ml aliquots of n-heptane and backwashed with 10% acetic acid and water. This treatment, as shown by zero time control studies, effectively removed all traces of the original labeled acetate. After concentration, aliquots of this extract were plated and counted directly (*non-volatile fatty acid radioactivity*). Acetoacetate was decarboxylated at end of incubation by addition of aniline citrate to the acidified medium(4). The $C^{14}O_2$ liberated, representing carboxyl-carbon, was collected in a KOH center well and assayed as for metabolic $C^{14}O_2$. Inhibitors† were made to the appropriate final concentrations in buffered medium as sodium salts. All radioassays were carried out using the windowless flow proportional counter of Robinson(5). Self-absorption corrections on $BaCO_3$ and cholesterol digitonide samples were made to the 3.1 mg/cm^2 point.

Results. In vitro studies with alpha-phenyl-n-butyrate. At 1 x 10^{-3} M alpha-phenylbutyrate strongly inhibited incorporation of sodium acetate-1-C^{14} into cholesterol (*non-saponifiable lipid*). In the typical experiment summarized in Table I the rate of incorporation in presence of the drug was only about one-half that of control. Extent of inhibition did not appear to be a function of the time of incubation. However, it did vary from rat to rat. In 6 experiments with incubation times ranging from ½ to 2 hours, inhibition varied from 32% to 62%, mean 42%. The reason for this variation, as well as for variation in rate of incorporation in normal control rats noted by many laboratories, is not entirely understood(6-8).

TABLE I. Effect of Alpha-phenylbutyrate on Acetate-1-C^{14} Incorporation into Lipids.*

Flask	Conc. of alpha-phenyl-butyrate	Total radioactivity in lipid fractions (10^4 c.p.m./g liver slices) Non-saponifiable lipid	Non-volatile fatty acids
1	0	1.06	.093
2	0	1.00	.088
3	1 × 10^{-3}M	.48	.030
4	"	.61	.040

* 30 min. incubation. 400-420 mg liver slices in 3.5 ml medium. 1 μc sodium acetate-1-C^{14} added (1 mc/mM).

Incorporation into non-volatile fatty acids was also inhibited to about the same extent. In 5 experiments it ranged from 23% to 62% with a mean of 41%.

The observed inhibition of both cholesterol and fatty acid synthesis from acetate suggested that the site of action of the drug might be at an early step common to both biosynthetic pathways. Incorporation of labeled acetate into acetoacetate was measured and, as shown in Table II, was also inhibited by about 50%.

At 1 x 10^{-3} M the drug did not significantly affect the endogenous oxygen consumption of liver slices (Table II), suggesting that the effects on lipid synthesis are not due to a basic disruption of the over-all tissue metabolism. This is further indicated for, in a number of experiments the oxidation of labeled acetate to $C^{14}O_2$ was decreased only by 5-15% (see Table II). Apparently the reactions of tricar-

TABLE II. Effects of Alpha-phenylbutyrate on Oxygen Uptake, Acetate-1-C^{14} Oxidation and Incorporation into Acetoacetate.*

Phenylbutyrate concentration	Total radioactivity (10^4 c.p.m./g liver slices) Acetoacetate	CO_2	Oxygen uptake (μl/g wet wt)
0	.80	2.34	927
1 × 10^{-3}M	.41	2.06	896

* 120 min. incubation. 430-480 mg liver slices in 3.0 ml medium. 0.1 μc sodium acetate-1-C^{14} added (1 mc/mM).

† Alpha-phenyl-n-butyric acid was purchased from Distillation Products Industries; α,α′-diphenylbutyrate and β,β′-diphenyl-α-ethylpropionate were synthesized in Dr. Evan C. Horning's laboratory; α,α′-diphenylvalerate and 1-dimethylamino-2,2 -diphenylpentane HCl were made by Smith, Kline and French Laboratories (SKF 2314 and SKF 3019 A).

TABLE III. Effects of Compounds Structurally Related to Alpha-phenylbutyrate on Acetate-1-C^{14} Oxidation and Incorporation into Non-saponifiable Lipid Fraction.

Inhibitor	Total radioactivity (10^4 c.p.m./g liver slices)			
	CO_2		Non-saponifiable lipid	
	Control	Inhibited	Control	Inhibited
α, α′-diphenylbutyrate (1×10^{-3}M)*	21.0	20.0	1.02	.36
β, β′-diphenyl-α-ethyl propionate (1×10^{-3}M)*	21.0	16.6	1.02	.44
α, α′-diphenylvalerate (1×10^{-3}M)†	24.3	12.2	.92	.17
Idem (1×10^{-5}M)‡	14.4	10.1	.031	.025

* 120 min. incubation. 500 mg liver slices in 5 ml medium. 1 μc (= 1 μM) acetate-1-C^{14} added.

† 60 min. incubation. 380-400 mg liver slices in 3.5 ml medium. 1 μc acetate-1-C^{14} added.

‡ 60 min. incubation. 400-410 mg liver slices in 3.5 ml medium. 150 μM unlabeled acetate with 1 μc (= 1 μM) of acetate-1-C^{14}.

boxylic acid cycle and reactions of terminal oxidation can proceed with little or no impairment in the presence of these concentrations.

Inhibition of $C^{14}O_2$ production, while on the average considerably less than inhibition of lipid synthesis (mean of 7 experiments 15%), was nevertheless highly significant. In 2 experiments this inhibition rose to about 40%. This finding, as discussed further below, suggests that there is inhibition either in activation of acetate(9) or in condensation reaction through which acetyl-coenzyme A (acetyl CoA) enters the tricarboxylic acid cycle(10). When the concentration of alpha-phenylbutyrate was raised to 1 x 10^{-2} M, incorporation of labeled acetate into cholesterol and fatty acids was almost completely inhibited and incorporation into acetoacetate was inhibited by more than 70%. However, at this concentration there was also inhibition of oxygen consumption, as much as 25% in some experiments, and inhibition of the rate of incorporation of alanine-1-C^{14} into liver proteins(11). At this higher concentration, then, the drug interfered with tissue respiration and presumably also with energy metabolism.

In vivo studies with alpha-phenyl-n-butyrate. It has been proposed(3) that alpha-phenyl-butyrate might interfere with cholesterol synthesis by forming an extremely stable CoA complex, thus reducing the effective levels of the coenzyme. The concentrations of CoA in livers of rats fed alpha-phenylbutyrate, 200 mg/kg/day for 4 weeks, were measured (12). The values found for control animals were: 163, 228, 179 units g; for the drug-fed animals, 203, 207, 226 units/g.‡ While the results in such a small group cannot be considered conclusive they suggest that any dramatic effects on levels of available CoA are not to be expected. Since the experiments of Klein and Lipmann(13) show that liver CoA must fall to 50 or 60 units/g before lipid synthesis is inhibited it is unlikely that availability of CoA is a limiting factor in lipid synthesis of phenylbutyrate-fed rats. Total liver cholesterol in these drug-fed animals also remained at normal levels.

In vitro studies with related compounds. Several compounds structurally related to alpha-phenylbutyrate have been studied *in vitro* in a similar manner. As shown in Table III, 2 of these (α,α′-diphenylbutyrate and β,β′-diphenyl-α-ethyl-propionate) have similar effects. At 1 x 10^{-3} M they inhibit cholesterol synthesis by somewhat over 50% without markedly inhibiting acetate oxidation. The third compound (SKF 2314 = α,α′-diphenylvalerate) is a considerably more potent inhibitor of cholesterol synthesis, effective even at concentrations of 1 x 10^{-5} M. These compounds, aromatic acids with structures close to that of alpha-phenylbutyrate, have been previously shown by Brodie *et al.*(14) and by Cook *et al.*(15) to inhibit a number of reactions in biotransformation of drugs. The spectrum of reactions inhibited is quite broad (*e.g.* hydroxylation, barbiturate side-chain oxidation, dealkylation) but they share in common a requirement for oxygen and triphos-

‡ We wish to thank Dr. Earl R. Stadtman for his assistance in the CoA analyses.

phopyridine nucleotide and localization in microsomes(13).

Studies by La Du *et al.*(16) have shown further that the carboxyl group is not an absolute structural requirement for inhibition in microsomal systems. 1-dimethylamino- 2,2-diphenylpentane hydrochloride (SKF 3019 A) proved extremely potent in inhibiting the dealkylation reaction. It was of interest, then, to test this compound in our system. At 1 x 10^{-3} M it inhibited acetate incorporation into cholesterol by 53% and acetate oxidation to CO_2 by 17%.

Discussion. These data suggest that the effect of alpha-phenylbutyrate on acetate incorporation into lipids represents inhibition at one of the earliest levels of acetate metabolism, probably before formation of aceto-acetate. The site of inhibition could be either: 1) in the formation of acetyl-CoA; or 2) in subsequent acetylation reactions involving acetyl-CoA; or 3) both.

The fact that incorporation into lipids was generally inhibited more strongly than oxidation to CO_2 does not rule out inhibition at the activation step. Since the reaction pathway to CO_2 is favored (Table III), the percentage inhibition in this direction, when the supply of acetyl-CoA is decreased, might well be less than the percentage inhibition in less favored pathway toward cholesterol and fatty acid synthesis.

Under 2) above are included key reactions of acetylation of oxalacetate to form citrate and acetylation of acetyl-CoA to form aceto-acetyl-CoA. A differential inhibition of these 2 acetylation reactions would account for the observation that CO_2 formation was inhibited less than incorporation into lipids. Inhibition of acetoacetate formation by ethylthioacetate and benzoate has been demonstrated by Avigan, Quastel, and Scholefield(17). They showed that these compounds probably exert their inhibitory effects as CoA derivatives. In a similar manner alpha-phenylbutyrate may inhibit only after formation of an acyl-CoA complex.

Whether *in vitro* inhibition of cholesterol synthesis from acetate described here represents the mechanism for *in vivo* effects on serum cholesterol levels reported by Cottet *et al.*(1-3) is not indicated by these studies and is the subject of further investigation.

The activity in our system of the drug potentiators studied by Brodie *et al.* is provocative. While the compounds may be acting in different ways in the 2 systems, further studies are called for to determine whether the manifold effects on drug metabolism may be mediated through effects related to the effects of alpha-phenylbutyrate on acetate metabolism.

Summary. 1) Alpha-phenyl-n-butyrate at 1 x 10^{-3} M has been shown to decrease rate of incorporation of acetate-1-C^{14} into cholesterol and into fatty acids by rat liver slices. 2) Incorporation of acetate into aceto-acetate is inhibited about the same extent, while oxidation to CO_2 is generally less strongly inhibited. 3) Endogenous oxygen consumption can proceed relatively unimpaired in the presence of 1 x 10^{-3} M alpha-phenylbutyrate. 4) Several compounds structurally related to alpha-phenylbutyrate are also demonstrated to inhibit acetate incorporation into lipids. 5) The possible relation between present observations on inhibition of lipid synthesis and studies of Brodie *et al.*(14) on drug potentiators is discussed.

1. Cottet, J., Redel, J., Krumm-Heller, C., and Tricaud, M. E., *Bull. Acad. Nat. de Med.*, 1953, No. 25-26-27, 441.

2. Bargeton, D., Krumm-Heller, C., and Tricaud, M. E., *C. R. Soc. de Biol.*, 1954, No. 1-2, 63.

3. Cottet, J., Mathivat, A., and Redel, J., *La Presse Medicale*, 1954, v62, 939.

4. Edson, N. L., *Biochem. J.*, 1935, v29, 2082.

5. Robinson, C. V., *Science*, 1950, v112, 198.

6. Fredrickson, D. S., Loud, A. V., Hinkelman, B. T., Schneider, H. S., and Frantz, I. D., *J. Exp. Med.*, 1954, v99, 43.

7. Tomkins, G. M., and Chaikoff, I. L., *J. Biol. Chem.*, 1952, v196, 569.

8. Langdon, R. G., and Bloch, K., *ibid.*, 1953, v202, 77.

9. Jones, M. E., Lipmann, F., Hilz, H., and Lynen, F., *J. Am. Chem. Soc.*, 1953, v75, 3285.

10. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, 1951, v191, 161.

11. Anfinsen, C. B., and Steinberg, D., *ibid.*, 1951, v189, 739.

12. Stadtman, E. R., Novelli, G. D., and Lipmann,

F., *ibid.*, 1951, v191, 365.

13. Klein, H. P., and Lipmann, F., *ibid.*, 1953, v203, 101.

14. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C., and Udenfriend, S., *Science*, 1955, v121, 603.

15. Cook, L., Macko, E., and Fellows, E. J., *J. Pharm. and Exp. Therap.*, 1954, v112, 382.

16. La Du, B. N., Horning, E. C., Wood, H. B., Trousof, N., and Brodie, B. B., *Fed. Proc.*, 1954, v13, 377.

17. Avigan, J., Quastel, J. H., and Scholefield, P. G., *Biochem. J.*, 1955, v60, 329.

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A Method for Quantitating Flexibility Changes of Pelvic Joints in Rats and Mice.* (21993)

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An increase in flexibility of the pelvic joints in mice occurs during pregnancy and following appropriate hormonal treatment(1-3). This increase was determined by manual manipulation of the pelvis. A more reliable method, suitable for both the mouse and rat, was devised to measure small differences which can be statistically analyzed.

Materials and methods. To quantitate changes in flexibility of the sacroiliac joints the animal is sacrificed, skinned, its body divided at the level of the iliac crests, and the tail severed at the 3rd caudal vertebra. The muscles, which arise in part from the dorsal aspect of the sacrum and 1st caudal vertebra to insert on the extremities, are severed at their origins and reflected laterally. The extremities and pelvic viscera are then removed. All of the musculature attached to the innominate bones is severed close to the bone, except the iliac origins of the sacrospinalis, the iliococcygei (abductor caudae internus), and the pubococcygei (abductor caudae externus; Fig. 1, PC) which are left intact. (Careless removal of the iliac origin of the sacrospinalis might damage the dorsal ligaments of the sacroiliac joint (Fig. 1, SI); the latter 2 muscles hold the ischial tuberosities in a convenient position for making subsequent measurements. Preliminary tests showed that the weight used to flex the sacroiliac joint produced maximum flexion whether or not these muscles were present.) A straight dissecting needle is inserted into the neural canal as far as it can go under moderate pressure (Fig. 1, C). The free end of the needle is slipped into an adjustable clamp which holds the specimen in a horizontal position 2 inches from a transparent plastic protractor (Fig. 1, B, J, M). The specimen's position is adjusted so that the zero line of the protractor is aligned with the dorsal-most portion of the left iliac crest and ischial tuberosity (Fig. 1, F′). To assure constant, correct alignment the positioning of the specimen is performed while viewing it through a hole (4 mm in diameter) in the center of an opaque rectangle (3 x 5 inches) placed 5 inches from the protractor (Fig. 1, A, L). A 50 g weight sufficient to produce a 3°-5° displacement of the ischial tuberosity in normal rats or a 2 g weight sufficient to produce a 1° displacement in normal mice is suspended from the symphysis pubis (Fig. 1, K). A raising of the protractor to realign the zero line with the dorsal-most portion of the iliac crest is necessary at this time because the crest is slightly elevated as the ischial tuberosity is lowered by the weight. While viewed through the hole in the rectangle a movable transparent plastic arm attached to the protractor is lowered by the observer's finger so that a black line running through the length of the arm aligns with the dorsal-most portion of the now displaced ischial tuberosity (Fig. 1, I, G′). Displace-

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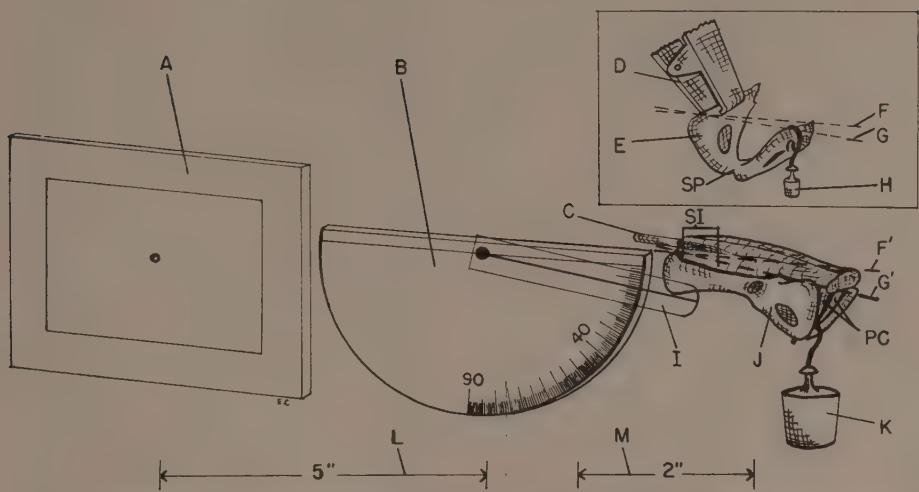

FIG. 1. Diagram of apparatus used for quantitating flexibility changes of pelvic joints in rats and mice. Relative dimensions of rat's pelvis are shown.

ment was measured in degrees at 30 seconds after the weight was applied by recording the position of the line of the arm in relation to the degree markings on the protractor.

To quantitate changes in flexibility of the symphysis pubis of the mouse (Fig. 1, SP) (interpubic synchrondrosis in the rat) the innominate bones are separated from the vertebral column by transecting the ilia close to the acetabula. The left ischium is placed in an adjustable clamp which holds the innominates in a horizontal position (Figs. 1, E; 2).

FIG. 2. Photograph of apparatus being used to quantitate flexibility of a rat's interpubic synchrondrosis.

Each ischial tuberosity is 2 inches from the protractor. While viewed through the hole in the rectangle the zero line of the protractor is aligned with the dorsal-most portions of both ischial tuberosities (Fig. 1, F). A weight sufficient to produce a 1° displacement in normal animals (1 g for rat, 0.1 g for mouse) is suspended from the right ischium midway between the tuberosity and the acetabulum (Fig. 1, H). Fifteen seconds from the time the weight was applied the amount of displacement of the right ischial tuberosity is recorded using the arm attached to the protractor in the same manner as described for determining sacroiliac joint flexibility (Fig. 1, G). Once adept, one can perform the entire procedure in less than 10 minutes from the time the animal is sacrificed.

Detailed reports in which this method was used to quantitate changes in pelvic joint flexibility in the rat and mouse during pregnancy and following estrogen and relaxin treatment are forthcoming.

Summary. A method was devised for measuring small differences in pelvic joint flexibility in rats and mice. The procedure in which weights are used to produce joint flexion and a protractor to measure the amount of flexion in degrees is described.

1. Gardner, W. U., *Am. J. Anat.*, 1936, v59, 459.
2. Crelin, E. S., *ibid.*, 1954, v95, 47.
3. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 22.

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Response of Mice to Standard Infecting Doses of *Mycobacterium tuberculosis* var. *hominis*.* (21994)

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We described(1) a procedure which could be used to determine the effectiveness of chemotherapeutic agents for the suppression of an experimental tuberculous infection of mice. Data available at that time indicated that the method was highly reproducible. This method now has been applied to the *in vivo* assessment of chemotherapeutic activity for a period of 5 years. Not only has the original estimation of the reliability of the method for this purpose been confirmed, but, in addition, data has been collected during this period which is of importance in regard to the response of untreated normal mice to tuberculous infection. One to 6 groups of untreated normal mice have been infected almost every month during this period. The nature of the response of these untreated control mice to inoculation with a standard infecting dose of *M. tuberculosis* var. *hominis* H37Rv will be the subject of the present communication.

* This study was aided in part by a grant from Parke, Davis & Co., Detroit, Mich.

Method. Since the standardized mouse infection procedure has already been described in detail(1) it will suffice here to state that "Strong A" strain mice weighing between 18 and 22 g were maintained and housed under standard conditions and infected intraven-

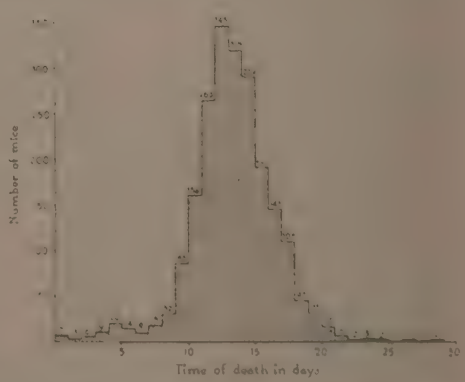

FIG. 1. Distribution of deaths of mice infected with 1.0 mg of H37Rv strain of *M. tuberculosis* var. *hominis*.

TABLE I. Median Survival Times of Groups of Strong A Strain Mice Injected Intravenously with 1.0 mg of the H37Rv Strain of *M. tuberculosis* var. *hominis*.

Year	Month												Yearly means
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	
1950		12.5	13.5	15.0	15.0	14.0	13.0			13.0	13.0	13.5	13.57
		13.0	14.5	16.0	14.0	14.0	13.5			12.5		11.5	
			14.0		14.0	13.5				11.5			
			15.0			12.5							
1951	12.5	12.0		14.5	15.5	11.5	14.5	15.0	12.5	14.5	12.5	14.0	13.53
	14.0	12.5		14.0	13.5	11.5		16.0	15.0	14.0	12.5		
	14.0					11.0		16.0	12.0	13.0	11.5		
						13.5				14.5	13.0		
										15.5			
1952	16.0	13.0	12.0	12.0	11.0	11.5	12.5	13.0	14.5	13.0	15.0	13.5	13.11
	14.0	14.5	12.5	14.5	12.5	14.5	14.5	10.0	14.5	14.0	12.5	10.5	
	14.0	13.5	11.0	12.5	12.0	13.5			10.5	11.5	11.5	15.5	
	13.0	14.5	15.0	13.0	14.0				12.5	13.5	14.5		
			12.0		13.5					13.5			
1953	15.5	13.0	11.5	14.5	12.5	12.5	13.0	12.5	13.0	13.5	13.0	14.5	12.70
	11.5	12.5	14.5	12.0	12.0	11.5	12.0		13.0	14.0	14.5	13.5	
	14.0	12.0		12.5	14.5	8.0	10.5		12.5	12.0		12.0	
	12.0	12.5		13.0	12.0	12.5	11.5		14.5	12.5			
					13.5		15.0			11.5			
					10.0								
1954	18.0	14.5	14.0	12.0	14.0	15.0	13.5	12.0	13.0	13.5	13.0	14.5	13.47
	14.0	13.5	13.0	12.0	13.5		12.5	10.5	13.5	12.0	14.0	12.5	
	13.5		12.5	13.5	14.0		11.5	12.5	11.0	14.5	14.5	12.0	
									15.0	19.5		14.0	
										12.0		14.0	
Monthly means	14.0	13.10	13.21	13.40	13.21	12.53	12.88	13.05	13.13	13.43	13.11	13.25	

ously with 1.0 mg wet weight of the virulent H37Rv strain of *Mycobacterium tuberculosis* var. *hominis*. Following inoculation a record was made of the time of death in days of each mouse. Each mouse was autopsied shortly after death in order to confirm that death was due to tuberculosis. The cumulative percent of mice dead was plotted against time of death on logarithmic probability paper, the median survival time estimated and the 95% confidence limits calculated using the method of Litchfield(2).

Results. Fig. 1 shows the distribution of deaths of 2,123 of the 2,173 untreated normal mice contained in the 180 groups which were infected over the 5-year period 1950 through 1954. The mean survival time of the mice shown in Fig. 1 was 13.84 ± 3.16 days. Fig. 1 does not include 4 mice which died at the time of inoculation; 37 mice which were accidentally killed, and 9 mice which lived more than 30 days. Inclusion of the 9 mice which lived longer than 30 days gives a mean survival time of 14.02 ± 4.56 days.

Table I shows the distribution of median survival times of the 180 groups of mice according to both year and month for the years 1950 to 1954 inclusive. The yearly means of the median survival times varied between 12.70 and 13.57 days. Analysis of variance showed that statistically these yearly means did not differ significantly. The means of the monthly median survival times varied between 12.53 and 14.0 days. Again, analysis of variance revealed that statistically these means did not differ significantly.

Discussion. The preceding data clearly show that with standardization of technic and by the use of a strain of *Mycobacterium tuberculosis* var. *hominis* maintained at constant virulence(3) the response of an inbred strain of mice to tuberculous infection can be remarkably uniform. The demonstration of such uniformity of response and the definition of the conditions necessary to produce it are highly desirable preliminaries to attempts to measure, on the one hand, the degree of natural or acquired immunity to tuberculosis or,

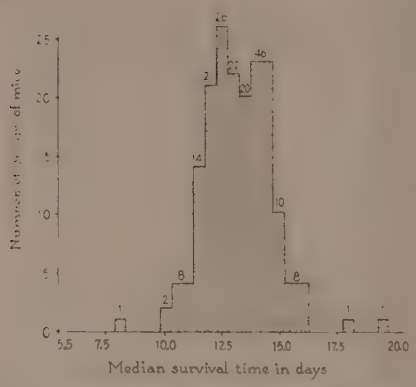

FIG. 2. Distribution of median survival times of groups of mice injected with 1.0 mg of H37Rv strain of *M. tuberculosis* var. *hominis*.

on the other, the virulence of strains of Mycobacteria.

The data also offer no support for the occurrence of any yearly or seasonal variation in the resistance of this strain of mice to tuberculous infection. This might in part be a reflection of the fact that these mice were housed in an air conditioned animal room and were therefore not subjected to extreme variations in temperature. However, this room faces south and during the summer temperatures in this room ranging between 90° and 95°F have been recorded. The observed extreme temperatures in this room have been approximately 65°F in the winter and approximately 95°F in the summer.

Summary. 1. Over a 5-year period, 1950 through 1954, one to 6 groups per month of 10 to 30 mice each were infected intravenously with a 1 mg inoculum of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis*. 2. The median survival times of 177 (98.3%) of the 180 groups infected during this period fell between the 10th and 16th day inclusive. Statistical analyses revealed that there was no significant difference between the yearly means of the median survival times nor was there significant difference between the monthly means of the median survival times.

1. Youmans, G. P., and Youmans, A. S., *Am. Rev. Tuberc.*, 1951, v64, 541.

2. Litchfield, J. T., Jr., *J. Pharmacol. and Exp. Therap.*, 1949, v97, 399.

3. Youmans, G. P., *Annals N. Y. Acad. Sci.*, 1949. v52, 662.

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Hypothalamic Lesions in Goldthioglucose Injected Mice.* (21995)

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(Introduced by F. J. Stare.)

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Recent investigations have demonstrated the essential similarity of goldthioglucose obesity and hypothalamic obesity in mice(1-3). This in turn led to the re-examination of the possibility that hypothalamic lesions may be involved in the mechanism of action of goldthioglucose despite the fact that other investigators(4,5) did not succeed in demonstrating such lesions. Since the possibility existed that through a process of reabsorption and repair, any damage to the central nervous system may be at least in part obscured in the obese animal during the period required for the obesity to develop, it was decided to examine injected animals shortly after goldthioglucose administration. Goldthiomalate, though similar to goldthioglucose in gold content, molecular weight and toxicity does not produce obesity(6), consequently it was de-

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† Life Insurance Medical Research Fund Predoctoral Fellow.

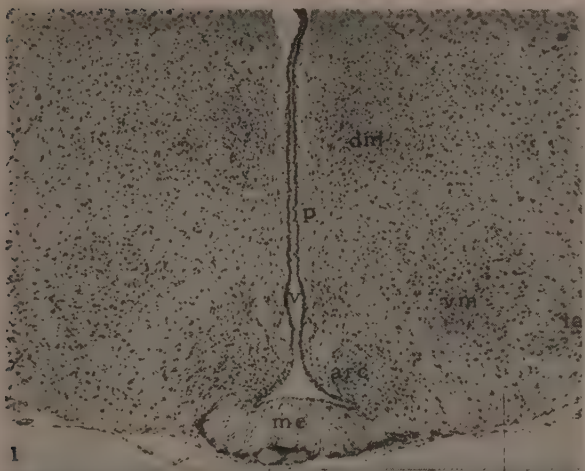

PLATE I: Photomicrographs (×65) taken of hematoxylin and eosin stained sections through tuberal or infundibular region of the hypothalamus showing the same area in 3 different animals.

FIG. 1. Section through hypothalamus of a goldthiomalate treated mouse, indicating a normal hypothalamic nuclear configuration and containing a normal number of neurons. Photomicrograph is labeled to indicate normal landmarks and nuclei seen in this region of the hypothalamus: dm, dorsomedial nucleus; f, fornix; p, periventricular region; v, third ventricle; vm, ventromedian nucleus; lat, lateral hypothalamic area; arc, arcuate nucleus; me, median eminence.

FIG. 2. Same area as in Fig. 1 of hypothalamus of mouse three days after the administration of goldthioglucose. Note marked loss of neurons and presence of pyknotic cells in ventral part of hypothalamus. Normal hypothalamic nuclear pattern has been lost. Arrows on right side of photomicrograph point to punctate hemorrhages. Arrow on left points to line of demarkation between area of edema (ventral) and normal tissue (dorsal).

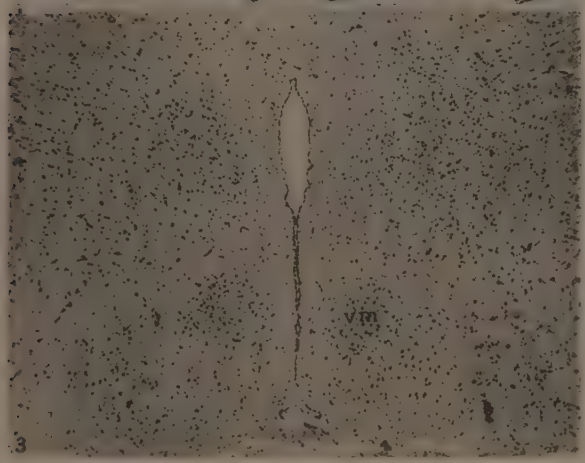

FIG. 3. Section of hypothalamus of obese mouse three months after the administration of goldthioglucose. Compare with Fig. 1 and 2. This section is slightly posterior to Fig. 1 (more ventral position of fornix (f)). Although there is no evidence of edema as in Fig. 2, there is an obvious decrease in the number of cells. The ventromedial nucleus (vm) can barely be made out as a discrete nuclear mass. The tissue forming the median eminence (bottom) has been destroyed.

cided to use goldthiomalate injected animals as controls.

Materials and methods. All the animals used were female Swiss mice averaging in age from 4 to 6 months. Out of 100 or more animals of each type, the following representative animals were examined: 6 uninjected control mice, 7 mice injected with goldthioglucose 24 to 72 hours prior to autopsy, 5 mice which had become obese as a result of goldthioglucose treatment, 4 mice which failed to become obese after being injected with goldthioglucose at least 3 months prior to autopsy, and finally 5 mice injected with goldthiomalate 24 to 72 hours prior to autopsy. The non-obese mice weighed 25 to 35 g; the obese mice weighed 45 to 65 g. Each of the treated mice was injected with 1 mg/g of body weight of the appropriate gold compound. This dosage represents a 50% lethal dose(6). Each brain was carefully dissected free of its bony enclosures and fixed for 24 hours in 10% neutral formalin. The overlying thalamus, subthalamus, and cerebral cortex were not trimmed and were used for control neural tissue in each specimen. The blocks of brain were dehydrated in a routine manner, embedded in paraffin and serial sectioned at 10 mu throughout the entire block. The sections were stained with hematoxylin and eosin and examined.

Results. Hypothalami of the goldthioglucose injected animals showed remarkable lesions within one to 3 days generally consisting of diffuse and punctate hemorrhages, widespread and marked edema involving more neural tissue than the hemorrhagic areas and pyknosis and degeneration of nerve cells throughout areas of edema and hemorrhage (Fig. 2, 4, 5, 7). The ventral portion of the third ventricle, especially the infundibular recess, also showed changes in that it was frequently engorged with blood and the lining ependymal cells were sometimes pyknotic and disrupted (Fig. 2, 4, 7). Although the anatomical extent and severity of the hypothalamic damage varied from animal to animal, it was clear in all cases that at least the ventral portion of the hypothalamus was involved. In the most severe instances, there was marked destruction of tissue at the base of the brain between the optic chiasm and the mamillary bodies, so that the neural tissue lying ventral and lateral to the third ventricle was fragmented and necrosed. In these specimens the usual landmarks in the ventral hypothalamus such as the supra-optic nucleus, ventromedial nucleus, median eminence, arcuate nucleus, and the lateral hypothalamic area were completely destroyed. In the less severe cases, the hypothalamic tissue at the base of the brain was intact, and the hypothalamic nuclei recognizable as clumps of cells, but the neurones in this area showed the signs of damage, and the parenchyma was edematous and hemorrhagic (Fig. 2, 4).

The limits of this edemous hypothalamic tissue were in all cases sharply demarkated (Fig. 2, 4, 5, 7). Dorsal to the edematous area, the neurones appeared normal. There was an abrupt transition from the normal tissue to the less eosinophilic edematous tissue that contained damaged neurones. The punctate and diffuse hemorrhagic areas were nearly always found within the edematous regions. They were most frequently found in the neural tissue adjacent to the ventral portion of the third ventricle and in the lateral hypothalamic area (Fig. 2, 4, 7).

Anteriorly, at the level of the optic chiasm, the damage appeared restricted to the most ventral nuclei adjacent to the chiasm. A bit posteriorly, the damage was more extensive, reaching more dorsally, so that in several specimens the paraventricular nucleus was involved, but at the same level, the laterally placed fornix was unaffected in all specimens. The supra-optic nucleus, the suprachiasmatic nucleus, and the ventral parts of the anterior and lateral hypothalamic areas were damaged in all specimens.

In the middle hypothalamic region (infundibular), the most extensive and severe damage was found. The ventromedial nucleus, the ventral part of the lateral hypothalamic area, the arcuate nucleus, and the median eminence were always destroyed (Fig. 2, 4, 5, 7). In 3 animals the dorsomedial nucleus and the perfornical area were also involved (Fig. 3, 5), but in no cases were regions lateral or dorsal to these damaged. In the posterior hypothalamus, only the mammillary bodies and

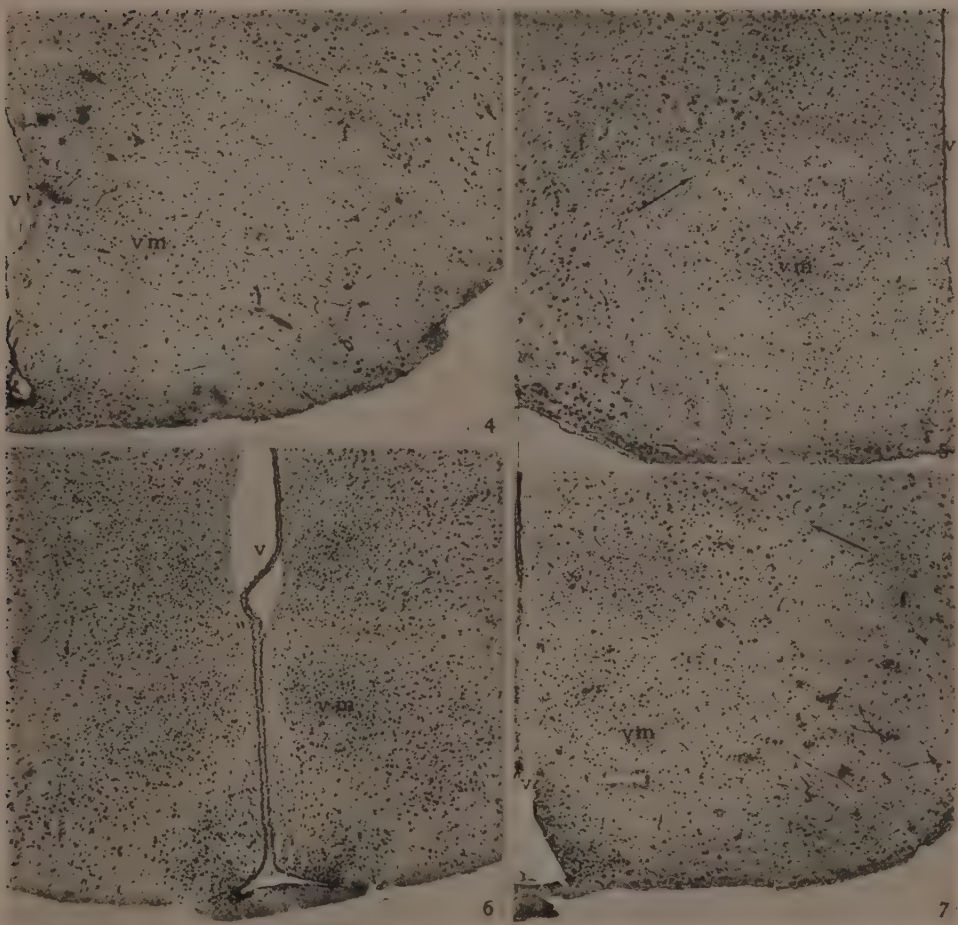

PLATE II: Photomicrographs (×65) taken of hematoxylin and eosin stained sections through tuberal or infundibular region of hypothalamus showing the same area in 4 different mice. Fig. 4, 5, and 7 were goldthioglucose treated. Fig. 6 was goldthiomalate treated. In Fig. 4, 5, and 7 note paucity of neurons in ventral hypothalamus, the pyknotic cell in edematous regions, and in Fig. 4 and 7, the widespread hemorrhages. In each figure position of the ventricle (v), ventromedial nucleus (vm), and fornix (f) is labeled. Arrows point to line of demarkation between destroyed (ventral) and normal (dorsal) regions.

the lateral area showed damage; the Fields of Forel and the cerebral peduncle outside the hypothalamus appeared normal.

Some inflammatory cells (polymorphonuclear leukocytes and lymphocytes) were present in the damaged areas, but their number was not great. Neurophagia and glial proliferations were observed, but these were scanty. In fact the large loss of neurons could not be accounted for in the 3 days after goldthioglucose administration by the amount of neurophagia observed. Presumably some of the neurons were lost by disruption and dissolution. There were no other areas of hemorrhage, cellular degeneration, and edema in the remaining parts of the brain overlying the hypothalamus.

The only lesion found in the brains of obese mice successfully injected with goldthioglucose at least 3 months prior to investigation was a paucity of neurons in the hypothalamus especially the ventral portion. This finding is

clearly demonstrable by a comparison of Fig. 1 and 3. The extent of the loss of neurons and replacement of glia was variable in the specimens examined. In one case this resulted in loss of discrete hypothalamic nuclear pattern which can be seen in the normal mice. In other specimens the nuclei could be recognized as clumps of cells, but they contained fewer neurons (Fig. 3).

Sections of the brain of animals given goldthiomalate showed nothing of the characteristic damage found in Swiss mice given goldthioglucose. In fact, as concerns the hypothalamus in particular, there were no differences from those of uninjected control mice. However, 2 of these animals had hemorrhages in the lateral ventricles, especially the frontal poles.

Examination of sections of brain of mice injected with goldthioglucose at least 3 months prior to autopsy and which failed to become obese revealed no distinct abnormality. The hypothalamic nuclei appeared distinct, and the number of neurons appeared within normal limits, though in some there seemed to be fewer neurons in a strip of hypothalamus lying ventral to the third ventricle.

The evidence presented here seems to support the conclusion that goldthioglucose produces obesity through causing hypothalamic lesions, always involving the ventromedial nuclei. Damage to the ventromedial nuclei has been shown to be essential to the production of obesity in rats(7) and mice(2). It is remarkable that, even though the hypothalamic area involved is often very large, only hyperphagia appears to result. The inactivity shown by hypothalamic mice(2) is not present in goldthioglucose obesity(6). Goldthioglucose obese mice do not show gonadal atrophy by weight(8).

Discussion. It would appear that the presence of the glucose component in the molecule makes this limited hypothalamic portion of the brain barrier much more permeable to this compound than to compounds not containing glucose. The hypothalamic area containing the feeding centers has been previously postulated to contain "glucoreceptors"(7). Larsson has since shown that this feeding center area effectively concentrates radioglucose with the receptivity apparently characteristic of the area rather than of discrete units(9). The results here would support the idea that the ventromedial feeding center area is a "glucoreceptive" area, in the sense postulated in the glucostatic theory of the regulation of food intake.

Summary. Goldthioglucose injections, in the dose which produces obesity in mice, cause in these animals extensive hypothalamic damage involving the ventromedial nuclei. Such lesions are permanent in animals which in fact become obese, but are not apparent in animals unsuccessfully injected. Goldthiomalate, though as toxic as goldthioglucose, does not cause obesity; its injection does not lead to hypothalamic lesions. Implications of these findings as regards the mechanism of the regulation of food intake are discussed.

The authors gratefully acknowledge the assistance of Dr. Charles DeWan. The goldthioglucose used in these studies as well as in previous studies was obtained through the courtesy of Dr. Edward Henderson and the Schering Corp., Bloomfield, N. J.

1. Bates, M. W., Zomzely, C., and Mayer, J., *Am. J. Physiol.*, 1955, v181, 187.

2. Mayer, J., French, R. G., Zighera, C. Y., and Barrnett, R. J., *ibid.*, 1955, v182, 75.

3. Mayer, J., and Zighera, C. Y., *Experientia*, 1955, v11, 358.

4. Brecher, G., and Waxler, S. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 489.

5. Owen, J. A., Jr., Parson, W., and Crispell, K. R., *Metabolism*, 1953, v2, 362, quoting unpublished investigations of Haymaker, W.

6. Marshall, N. B., and Mayer, J., *Am. J. Physiol.*, 1954, v178, 271.

7. Mayer, J., *Physiol. Rev.*, 1953, v33, 472.

8. Marshall, N. B., and Mayer, J., to be published.

9. Forssberg, A., and Larsson, S., *Acta Physiol. Scand.*, 1954, v32, suppl. 115 (Part 2).

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Prevention of Cobalt-Induced Polycythemia in Rats by Calcium Ethylene Diamine Tetra Acetic Acid. (21996)

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Polycythemia results from oral or parenteral administration of cobalt(1-5). It is known that this hematopoietic action of cobalt can be prevented by simultaneous feeding of sodium salt of ethylene diamine tetra acetic acid(6,7) and it is probable that under these circumstances cobalt is chelated in the gastro-intestinal tract preventing its absorption. As EDTA administered either as sodium or calcium salt rapidly combines with cobalt to form non-ionizable complexes, it is believed that administration of either of these compounds by the same route as cobalt does not effectively measure prevention of a polycythemic response to cobalt(8,9). It was of interest in this study to determine if EDTA administered as calcium salt prevents production of polycythemia when administered by a different parenteral route than cobalt.

Materials and methods. Six normal female rats of the Long-Evans strain, weighing 225 to 270 g, received daily intraperitoneal injections of 0.5 ml of a 1% saline solution containing 0.125 mg of cobalt as cobaltous sulfate. The injected solution also contained 0.200 mg of iron as ferric chloride to prevent iron shortage due to increased synthesis of hemoglobin under the influence of cobalt(10). Another group of 6 rats received daily 50.0 mg of CaEDTA subcutaneously in addition to the intraperitoneally administered cobaltous sulfate and ferric chloride. Red cell counts, hemoglobin, and hematocrit determinations were made after 34 days, and injections were continued for a total of 74 days, at which time red cell volumes were also determined. Six rats were retained without treatment to serve as controls. Blood samples were withdrawn under ether anesthesia from the external jugular vein into heparinized syringes. Hemoglobin concentrations were determined by the Turner method(11). Hematocrits were determined by Van Allen or Wintrobe tubes after centrifugation at 3,000 r.p.m. for 30 minutes. Blood volumes were determined by the Fe^{59}-labeled red cell method(12).

Results. Rats receiving cobalt showed significant increases in red cell count, hematocrit, hemoglobin, and total red cell volume, whereas no increase was found in rats injected with both cobalt and CaEDTA (Table I).

This experiment confirms the effectiveness of the amounts of cobalt and iron previously reported as capable of producing polycythemia in the rat(13). CaEDTA even when administered by a different parenteral route than cobalt still prevented the development of polycythemia by the cobalt.

Preliminary experiments, in which radioactive cobalt (Co^{60}) was used to determine the rate of excretion, showed that cobalt was excreted at a more rapid rate when CaEDTA was administered simultaneously.

Summary. Cobalt injected intraperiton-

TABLE I. Haematological Data in Rats Injected with Cobalt, Alone or in Combination with CaEDTA. Six rats in each series.

Treatment	Days inj.	Body wt, g	Hemoglobin, g/100 ml	Hemoglobin, g/100 g body wt	Hematocrit, %	Red blood cells $\times 10^6/mm^3$	Red blood cell vol/100 g body wt, ml
Cobalt	0	252	—	—	—	7.6 ± .3	—
	34	255	16.9 ± .8	—	64.3 ± 4.9	10.7 ± .6	—
	74	278	16.0 ± .5	.831 ± .033	63.0 ± 4.1	—	3.25 ± .20
Cobalt + CaEDTA	0	248	—	—	—	7.6 ± .1	—
	34	258	14.7 ± .7	—	47.6 ± 1.7	8.2 ± .4	—
	74	273	13.9 ± .3	.632 ± .018	50.0 ± 1.6	—	2.27 ± .09
Control	34	225	15.0 ± .1	—	48.8 ± .8	8.4 ± .4	—
	74	276	13.0 ± .5	.592 ± .027	48.0 ± 1.1	—	2.25 ± .09

eally into rats receiving calcium ethylene diamine tetra acetic acid subcutaneously did not produce the usual polycythemic response.

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1. Waltner, K., *Arch. Exp. Path. Pharmakol.*, 1929, v141, 123.

2. Waltner, K., and Waltner, K., *Klin. Wochsch.*, 1929, v8, 313.

3. Berlin, N. I., *J. Biol. Chem.*, 1950, v187, 41.

4. Garcia, J. F., Van Dyke, D. C., and Berlin, N. I., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 472.

5. Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *ibid.*, 1931, v29, 174.

6. Child, G., and Leonard, P., *Fed. Proc.*, 1951, v10, 286.

7. Child, G., *Science*, 1951, v114, 466.

8. Foreman, H., Vier, M., and Magee, M., *J. Biol. Chem.*, 1953, v203, 1045.

9. Martell, A. E., personal communication.

10. Van Dyke, D. C., Asling, C. W., Berlin, N. I., and Harrison, R. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 488.

11. Turner, A., *Bull. U. S. Army Med. Dept.*, 1946, v5, 605.

12. Berlin, N. I., Huff, R. L., Van Dyke, D. C., and Hennessy, T. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 176.

13. Berlin, N. I., Ph.D. Thesis, Univ. Calif., June, 1949.

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Total Cardiac By-pass Utilizing Continuous Perfusion from Reservoir of Oxygenated Blood.* (21997)

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The goal of our cardiac research efforts has been the development of simple, safe, and effective technics for direct vision intracardiac surgical procedures. The demonstration that total cardiac by-pass with right ventricular cardiotomy and septal suture can be successfully performed even in seriously ill patients by utilizing controlled cross circulation(1-4) doubtless will stimulate further rapid developments in this field.

This experimental study has been carried out on dogs to evaluate a method of substituting arterial and venous reservoirs of blood for the donor in the cross circulation set-up. By such a plan, it would be possible to perform intracardiac operations in a dry heart without the need of a donor in the operating room. Such a scheme immediately poses problems of procurement and storage of blood to supply the arterial reservoir. In this present experimental study intended to develop a feasible technic for total cardiac by-pass utilizing the reservoir method of perfusion, the arterial blood was obtained prior to the intended perfusion from a donor used as an oxygenator entirely independent of the perfusion set-up. The use of arterialized venous blood to supply the arterial reservoir has been described elsewhere(5).

Method. Collection. Ordinary 1,000 cc plasma vac§ bottles were used as reservoirs. These containers were readied for use by instilling in each 100 cc 5% dextrose and water solution containing heparin (25 to 32 mg) and at the same time exhausting the vacuum completely. Arterial blood was collected into these bottles (while in the inverted position) by gravity using a commercially available plastic collection set¶ connected to a plastic cannula

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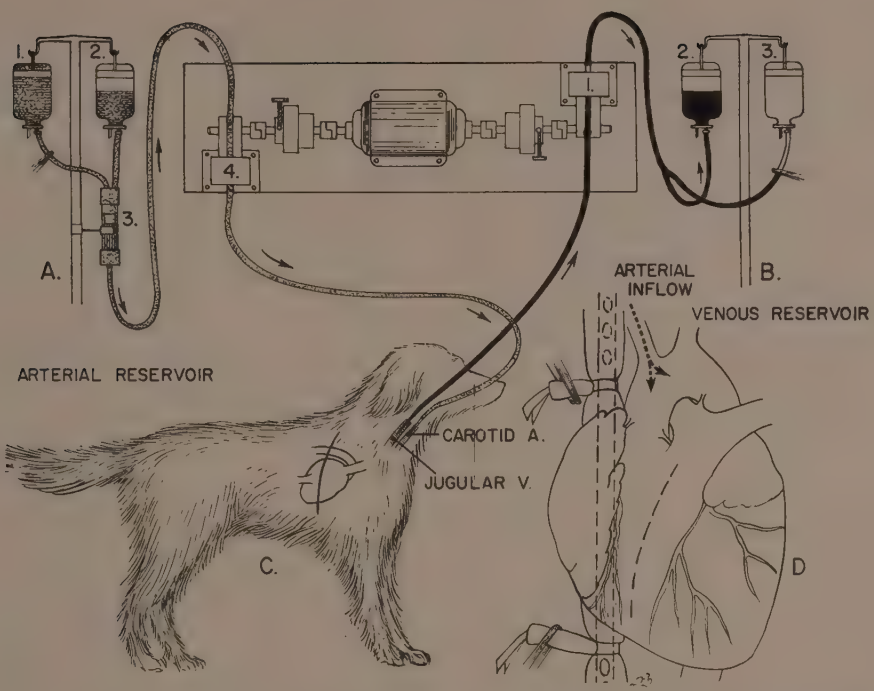

FIG. 1. Arterial reservoir perfusion. A. Arterial reservoir showing bottles of arterial blood (1 & 2), blood filter (3), and arterial pump (4). B. Venous portion of perfusion circuit with venous pump (1) and bottles (2 & 3) for collection of venous blood withdrawn from the patient's caval system. C. Recipient's relationship to the system is depicted in center of diagram and site of cannulations of right carotid artery and jugular vein are shown. Arterial cannula is inserted 3-4 cm down the carotid artery and ligated in place. Venous cannula with holes at tip and along shaft (the distance between these 2 sets of holes is determined by the space between the vena caval occluding tapes of the animal concerned) is passed down the jugular vein into the venae cavae until the holes are in the proper relation to the occluding tapes as indicated in D. At top center is pumping assembly which consists of separate pumps at each end driven by a single motor (center) and separate speed changers which are interposed between the motor and each pump head.

that had been placed in the donor's femoral artery. This cannulation in the donor animal was ordinarily done under pentothal sodium anesthesia or curare paralysis to permit endotracheal intubation for artificial respiration with 100% oxygen during the period of collection. However, in an occasional trained or cooperative animal cannulation was possible with only local procaine anesthesia. Synchronously with the withdrawal of arterial blood from the donor animal stored venous blood was infused into the donor's femoral vein by means of a cannula similar to that in the artery. To avoid any undesirable alterations in the donor's blood volume this venous infusion was maintained at a rate equal to the removal of arterial blood. The quantity of arterial blood withdrawn from the donor animal was dictated by the desired rate and duration of perfusion and usually ranged between 2000 to 3500 cc. After collection, the arterial blood was ordinarily placed in a water bath maintained at 39°C where it remained until needed for the perfusion. However, when there was to be a longer time interval between collection and perfusion the blood was kept refrigerated (at 4°C). In such instances it was rewarmed to 39°C before it was used for perfusion. *Operative method.* Mongrel dogs weighing from 4.4 to 11.5 kg were used as re-

cipient animals. All were premedicated with morphine 3-4 mg/kg of body weight and atropine gr. 1-100, and then anesthetized with 2½% solution of pentothal sodium injected intravenously. Endotracheal tubes were inserted, and except for the period of perfusion the animals were artificially respired throughout the operative procedure. Details of the methods of arterial and venous cannulations, vena cava occlusion, connections to the pump[¶] are depicted in Fig. 1 and were identical in every respect to those of the recipient animals in the cross circulation study group reported in detail elsewhere(1). The rates of perfusion were calculated on the basis of the reduced flow principle(6) and ranged between 25 to 30 cc/kg of recipient body weight/min. The length of perfusion was arbitrarily set at 20 minutes, during which time total cardiac and pulmonary by-pass was in effect. *Reservoir perfusion* (Fig. 1). As indicated above, the patient's (or recipient's) relationship to the extracorporeal circuit (C & D) is unchanged from that employed for cross circulation. The donor, on the other hand, has been replaced by containers of arterial blood (A 1 & 2) and empty venous receptacles (B 2 & 3) respectively. The 2 bottles of arterial blood were connected by means of a Y to a blood filter (A3) similar to those used for infusions of blood on the hospital wards. Blood was allowed to fill the filter system with the latter inverted so that all air was displaced from the chamber. The outflow of the filter chamber was then connected to the plastic tubing leading to the arterial pump (A4). After these connections were made, the pump was turned on for a few moments to completely fill the arterial circuit before the arterial outflow limb of the pump was connected to the arterial cannula (C & D) of the recipient. With this done, the arterial circuit was complete and after the recipient's caval cannula was connected to the tubing leading to the venous pump (B) perfusion was begun. Blood was withdrawn from the recipient's vena cavae via the cannula (C & D) connected to the plastic tubing by the venous pump (B1) and was collected into either venous receptacle (B2 or 3). In practice the arterial pump drew blood from only one bottle at a time and was replaced by another full one so that at all times there was an uninterrupted perfusion. Similarly when the venous reservoir (B2) was full, the venous outflow was diverted into the empty reservoir (B3) and (B2) was replaced by another empty bottle. The output from each pump was preset before the perfusion at the rate desired. Usually for the study animals these were set equal. However, occasionally when blood loss from a cardiotomy was anticipated an imbalance was intentionally created by reducing the venous pump rate below that of the arterial.

¶ Sigmamotor Inc., Middleport, N. Y.

TABLE I. Changes in Donor Hemoglobin and Platelets.*

Hemoglobin	
Control	13.6 g %
Final	12.8
Platelets	
Control	512,000
Final	365,000

* Avg figures for 10 donors, 3000 cc arterial blood was withdrawn from the femoral artery of each synchronously with infusion of equal amount of venous blood into femoral vein over a 1-2 hr period.

Observations. A total of 34 animals was used in this study (17 as recipients and 17 as oxygenators) to evaluate and develop this method of reservoir perfusion. Certain physiologic determinations were made on both the recipient and blood donor animals in an attempt to assess the alterations imposed by the method.

Hemoglobin and platelet alteration in the donor. Blood samples were taken from the donor's femoral vein cannula for hemoglobin and platelet determinations just before and after the collection of the arterial blood. The changes in 10 donors are tabulated in Table I. In these animals 3,000 cc of arterial blood was withdrawn from the femoral arteries while a like amount of citrated dog blood was administered into the femoral vein over a period of approximately one to 2 hours. The infused blood had been obtained from several dogs at sacrifice or as the by-product of a previous perfusion and had been stored for periods up to 5 days. Despite the varying quality and age of this infused venous blood the altera-

TABLE II. Hematologic and Metabolic Changes* Produced in Recipient after Reservoir Perfusion for 20 Min.

Recipient	pH	O_2 (vol %)	CO_2 (vol %)	Hgb. (g)	Platelets per mm^3
Control					
Artery	7.48	15.33	34.16	—	—
Vein	—	10.93	41.94	11.2	317428
Final					
Artery	7.26	18.53	30.67	—	—
Vein	—	12.03	38.96	13.4	182000

* The above are mean figures for 8 animals. The apparently low control arterial O_2 content is consistent with the avg control hemoglobin level (see text).

tions noted were relatively insignificant. In most of the donors the hemoglobin drop was approximately one-third of their normal level.

Changes in the recipients. In Table II are summarized the results of pH, O_2, CO_2, hemoglobin and platelet determinations made on 8 of the recipients just before and immediately after reservoir perfusion during which total cardiac by-pass was in effect. The most pronounced effect noted was the reduction in pH which in this instance fell from an average preperfusion level of 7.48 to 7.26 immediately postperfusion. This acidosis was similar to the observations made in total cardiac by-pass by other methods in studies previously reported(1,7,8) and probably was also due to the accumulation of acid metabolites during the perfusion period. The blood oxygen and carbon dioxide content determinations showed very little change. On first inspection the average control arterial O_2 content would appear to be low. However, this figure is consistent with the low control hemoglobin levels. The finding of an anemia in new stock animals admitted to the colony has not been unusual especially during the winter months. The rise in hemoglobin and its resultant increase in arterial O_2 content at the end of the perfusion was comparable to the hemoconcentration noted in previous perfusion experiments(7,8). The average blood platelet count fell to approximately one-half of its control level. The greatest drop in platelets demonstrated by any of the recipient animals was one-third of the control figure and ordinarily the recovery was relatively prompt so that the postoperative count was usually doubled by the following day and was back to its control level within 2 to 3 days.

There was no donor mortality in this study. During the early phases before the optimum heparin dosage had been established postoperative bleeding in the recipients was a frequent complication, and 6 of the first 7 recipients succumbed to this complication. However, of the last 10 recipients who received blood collected in less heparin (25-32 mg/liter) this complication has not occurred and 8 were long term survivors. The 2 deaths resulted from avoidable errors; one from avulsion of a carotid artery during decannulation and one from pulmonary edema caused by the inadvertent rapid infusion of a large amount of saline in the early postoperative phase.

Discussion. The results herein reported indicate that the use of the described method of continuous arterial perfusion from a reservoir of oxygenated blood is a feasible means for totally by-passing the heart and lungs for a time interval sufficient to perform intracardiac operations under direct vision. By the removal of the donor from the operating theater the treatment of patients with intracardiac abnormalities would be further simplified by a reduction in the number of personnel and the amount of equipment necessary. This method of collection of arterial blood from the donor with synchronous replacement by an equal quantity of compatible venous blood into the same body region would appear to be a simple and safe procedure for the donor associated with about the same risk as a conventional blood transfusion. If the blood is collected within a short time of its intended use the recipient (or patient) would appear to derive almost the same physiological benefits as when the donor is interposed directly into the extracorporeal circulation.

An obvious limiting factor inherent in this reservoir type of perfusion for clinical use is the fact that the length of the perfusion is limited by the amount of arterial blood collected for the reservoir. In practice several factors would appear to minimize this objection. First, clinical experience has confirmed the safety and practicability of total body perfusions at normal temperature utilizing re-

duced flow rates (25 to 40 cc of blood/kg body weight/minute). Secondly, experience gained in the corrective surgical treatment of intracardiac defects by the use of controlled cross circulation has indicated that, with direct vision, the majority of ventricular defects required well under 20 minutes of intracardiac operating time. These two factors coupled with the obvious fact that many of the urgent candidates for intracardiac operations are infants or small children make the technic herein described attractive for further investigation and clinical application.

In the present study the donor animal was used as an oxygenator for the procurement of arterial blood with which to supply the arterial reservoir. It is interesting to note that there was a relatively small depreciation in the donor's hemoglobin and platelets even though he "processed" a volume of blood which was usually greater than his total blood volume over a relatively short period of time. Also, this occurred in spite of the somewhat dubious quality of the stored venous blood infused into the donor dogs. This observation attests to the efficacy of the dog's hematopoietic regenerative powers and it would be reasonable to anticipate even less depreciation in these factors clinically with the use of a better quality of bank blood for the replacement.

Experimentally it was usually necessary to anesthetize the donor animal for the collection of arterial blood, but this would not be required in the clinical application of the method. Under these conditions, the collection could be performed via an arterial puncture of several compatible donors or from a single donor using local procaine infiltration of the puncture sites.

Summary. 1. A simple method permitting total by-pass of the heart and lungs for the performance of intracardiac surgical operations is described. 2. This method utilizes continuous perfusion of the recipient's (patient's) arterial system from a reservoir of oxygenated blood. An equivalent quantity of venous blood is removed from the recipient's superior and inferior cavae. 3. A pump is utilized to control this exchange between the recipient and the arterial and venous reservoirs. 4. Arterial blood for the reservoir was obtained from a donor animal used as an oxygenator independent of the perfusion system. This was done by collecting arterial blood from the donor's femoral artery into a heparin glucose solution while stored venous blood was infused into his femoral vein at an equal rate. Ordinarily 2,000 to 3,500 cc of arterial blood was collected from a single donor dog in this fashion. 5. The optimum dose of heparin to prevent clotting in the arterial blood collected for this purpose was 25 to 32 mg/liter of blood. 6. The simplifications of the clinical application of this method are briefly discussed.

1. Warden, H. E., Cohen, M., Read, R. C., and Lillehei, C. W., *J. Thor. Surg.*, 1954, v28, 331.

2. Lillehei, C. W., *Postgrad. Med.*, 1955, v17, 388.

3. Lillehei, C. W., Cohen, M., Warden, H. E., and Varco, R. L., *Surgery*, 1955, v38, 11.

4. Lillehei, C. W., Cohen, M., Warden, H. E., Ziegler, N., and Varco, R. L., *Surg., Gynec., and Obstet.*, Oct., 1955.

5. Warden, H. E., Read, R. C., DeWall, R. A., Aust, J. B., Cohen, M., Ziegler, N., Varco, R. L., and Lillehei, C. W., *J. Thor. Surg.*, in press.

6. Cohen, M., and Lillehei, C. W., *Surg., Gynec., and Obstet.*, 1954, v98, 225.

7. Cohen, M., Warden, H. E., and Lillehei, C. W., *ibid.*, 1954, v98, 523.

8. Warden, H. W., Cohen, M., DeWall, R. A., Schultz, E. A., Buckley, J. J., Read, R. C., and Lillehei, C. W., *The Surgical Forum, American College of Surgeons*, W. B. Saunders Co., Philadelphia, 1954, pp. 22-28.

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Effect of Sex Hormones on Conversion of Folic Acid to Citrovorum Factor by Rat.* (21998)

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The stimulatory influence of folic acid (PGA) and an inhibitory effect of aminopterin on the tissue growth response to estrogen in the female chick and rat was first reported by Hertz and Tullner(1). Gelfant *et al.*(2) reported that citrovorum factor (CF) partially reverses the inhibitory effect of aminopterin on estrogen induced mitoses in the rat uterus. Aminopterin is reported(3) to inhibit the urinary excretion of CF by rats receiving PGA.

The present report concerns the study of the effect of sex hormones on the *in vivo* conversion of PGA to CF by male rats.

Methods. Adult male rats of the Sprague-Dawley strain were placed individually in metabolism cages equipped with metal collecting funnels. Rats were maintained on Purina laboratory chow (Ralston Purina Co.) starting at the weaning stage and throughout the experimental period. Three separate experiments were conducted. In Exp. 1, 9 rats were randomized into 3 groups of 3 rats each. Group 1 received the Purina laboratory chow unsupplemented. Groups 2 and 3 received a daily supplement of 100 γ PGA by stomach tube. In addition to the latter, Group 3 received intramuscular injections of 7.7 γ estradiol dipropionate in sesame oil. In Exp. 2, 19 rats were randomized into 6 groups, 5 of which consisted of 3 rats each while Group 4 comprised 4 rats. Group 1 received the basal diet unsupplemented. Groups 2, 3 and 4 received a daily supplement of 100 γ PGA by stomach tube. In addition to PGA, Groups 3 and 4 received daily intramuscular injections of 0.5 mg testosterone and 10 γ estradiol dipropionate respectively. Groups 5 and 6 were controls for groups 3 and 4 and received daily intramuscular injections of 0.5 mg testosterone and 10 γ estradiol dipropionate respectively. In Exp. 3, 19 rats were randomized into 5 groups, 4 of which consisted of 4 rats each with the exception of Group 1 which had 3 rats. Group 1 received the basal diet unsupplemented. Groups 2, 3, 4 and 5 received a daily supplement of 100 γ PGA by stomach tube. In addition to PGA, Group 3 received daily intramuscular injections of 0.5 mg testosterone, Group 4 received daily intramuscular injections of 10 γ estradiol dipropionate, while Group 5 received daily injections of same amounts of both the sex hormones. Urine collections were started 24 hours after the rats had been maintained on the respective diets and were continued daily for a one to 2-week test period. The 24-hour urine collections from the rats of the same group were combined and an aliquot of the latter was filtered, neutralized and autoclaved for 30 minutes at 120°C. The heated samples were cooled, reneutralized, diluted and assayed. The CF content of the urine samples was determined by using the Bacto-Cf Assay Medium (Difco Laboratories). *Leuconostoc citrovorum* ATCC 8081† was used as the test organism, and Leucovorin (Lederle) was employed as the standard. The cultures were incubated 48 hours at 37°C and growth was measured by turbidimetric procedure. Since leucovorin has been reported to be half as active as the CF isolated from a horse liver(4), the microbiological assay values were divided by 2 in order to express the results in terms of

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† A recent report (Felton, E. A., and Niven, C. F., *J. Bact.*, 1953, v65, 482) concerning a taxonomic study on the culture *Leuconostoc citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae* as described earlier by Pederson (*Bact. Rev.*, 1949, v13, 225).

TABLE I. Effect of Sex Hormones on Urinary Excretion of Citrovorum Factor by Rats Receiving PGA.

Exp.	No. of rats	Supplement* to basal/day	Urinary excretion of CF, mγ/rat/day 1st wk	2nd wk
1	3	0	135	
	3	PGA, 100 γ	750	
	3	*Idem* + EDP,† 7.7 γ	1125	
2	3	0	155	
	3	PGA, 100 γ	785	
	3	*Idem* + Test.,† .5 mg	810	
	4	" + EDP, 10 γ	1320	
	3	Test., .5 mg	75	
	3	EDP, 10 γ	230	
3	3	0	232	170
	4	PGA, 100 γ	902	1068
	4	*Idem* + Test., .5 mg	1015	960
	4	" + EDP, 10 γ	1320	1454
	4	" + Test., .5 mg + EDP, 10 γ	1518	1495

* PGA administered by stomach tube while sex hormones were given by intramuscular injections.
† EDP = Estradiol dipropionate in sesame oil.
Test. = Aqueous suspension of testosterone.

the naturally occurring CF. At the end of the test period the rats from Exp. 2 were sacrificed, accessory sex organs and the testis removed, blotted and weighed.

Results. Administration of 100 γ PGA to male rats receiving a practical diet increases 4 to 6-fold the urinary excretion of CF (Table I). This increase is enhanced 50% by daily administration of 7.7 γ or 10 γ of estradiol dipropionate. On the other hand daily administration of 0.5 mg testosterone to male rats receiving PGA does not increase the urinary excretion of CF. The stimulatory effect of estradiol dipropionate on the CF excretion is not reversed by simultaneous administration of testosterone. It is interesting to note that rats receiving estradiol dipropionate alone also show a 50% increase in CF excretion, while the rats receiving testosterone alone show a 50% decrease in CF excretion.

It is apparent from the results in Table II that administration of 100 γ of PGA to the male rats does not influence the weight of the accessory sex organs or the testis. Estradiol dipropionate alone or together with PGA markedly decreases the size of the accessory sex organs and the testis. On the other hand testosterone alone or in combination with PGA significantly increases the size of the accessory sex organs.

Discussion. The results presented indicate clearly that *in vivo* conversion of PGA to CF, as measured by the urinary excretion of CF after administration of PGA is enhanced by estradiol dipropionate but is not influenced by administration of testosterone. It is interesting to note that the stimulatory effect of estradiol dipropionate on CF excretion is not reversed by the simultaneous administration of testosterone. Administration of 10 γ estradiol dipropionate alone gives a 50% increase in CF excretion, while the latter decreases by 50% on administration of 0.5 mg testosterone. Estradiol dipropionate markedly decreases while testosterone increases the size of the accessory sex organs. Under these conditions supplementing of PGA does not influence the size of the accessory sex organs. The administration of ascorbic acid to rats receiving PGA has been reported to enhance the urinary excretion of CF(3). Dietary homocysteine and cysteine enhance the urinary excretion of CF by rats receiving PGA

TABLE II. Effect of PGA and Sex Hormones on the Accessory Sex Organs and Testis in the Male Rat.

Supplement* to basal/day	Final body wt, g	Accessory sex organs† Seminal vesicle	Coagulating gland	Ventral prostate	Dorsal prostate	Testis
		mg/100 g				
0	342	108	36	131	65	444
PGA, 100 γ	361	100	45	150	68	452
Idem + Test., .5 mg	352	173	99	236	135	452
" + EDP, 10 γ	312	45	12	23	21	356
Test., .5 mg	354	176	75	262	153	454
EDP, 10 γ	304	51	14	21	12	329

* PGA administered by stomach tube while sex hormones were given by intramuscular inj.
† The figures denote mg of organs/100 g body wt.

(5). Extensive studies on the effects of dietary factors on the CF excretion have not been reported. A very interesting aspect of folic acid deficiency in the rat is the diminished response to estradiol induced uterine growth(1). An interference with metabolism of C^{14} labeled estrone in aminopterin-treated rats is reported by Trunnell *et al.*(6).

Summary. Administration of estradiol dipropionate alone or together with PGA enhances the urinary excretion of CF by male rats. Testosterone alone gives a 50% decrease in the CF excretion but is without significant effect when given in combination with PGA. The stimulatory effect of estradiol dipropionate can not be reversed by testosterone in the amounts tested.

1. Hertz, R., and Tullner, W. W., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1260.

2. Gelfant, S., Meyer, R. K., and Ris, H., *J. Exp. Zool.*, 1955, v128, 219.

3. Welch, A. D., Nichol, C. A., Anker, R. M., and Boehne, J. W., *J. Pharmacol. Exp. Therap.*, 1951, v103, 403.

4. Keresztesy, J. C., and Silverman, M., *J. Am. Chem. Soc.*, 1951, v73, 5510.

5. Doctor, V. M., and Trunnell, J. B., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 498.

6. Trunnell, J. B., Bowman, W. E., and Hildebrandt, H. E., Abstract, *J. Clin. Endocrinol. and Metab.*, 1954, v14, 796.

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Mechanism of Protective Effect of Hydrocortisone in Staphylococci Infected Adrenalectomized Mice.*† (21999)

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The reduced natural resistance of Addisonian patients as well as of adrenalectomized animals is a general phenomenon not specific for any one type of infection or intoxication. Although the mechanism by which adrenalectomy influences resistance remains relatively obscure, replacement therapy with adrenal cortical hormones has been shown to be beneficial(1,2). On the other hand, adrenal hormones may be detrimental to host resistance(3). It has been suggested, however, that the beneficial effects of cortisone therapy are to be derived only from dosages based on requirement(4,5). In this regard, Robinson, *et al.*(6), have recently pointed out that the detrimental effect of cortisone in resistance to pneumococcal infection in rats is primarily due to overdosage with the hormone, and that an optimal dosage schedule of cortisone can significantly enhance the host resistance of either adrenalectomized or intact infected animals. Hill, *et al.*(7), have described similar findings with systemic moniliasis in adrenalectomized mice treated with cortisone. Germuth, *et al.*(8), found that cortisone-treated rabbits could successfully resist an intravenous inoculum of *Staphylococcus aureus* which was fatal to approximately 50% of the untreated controls. They noted that the bacteria rapidly disappeared from the blood after injection in either treated or untreated animals. Kleiger and Blair(9) have described the acute death of experimental animals resulting from an inoculation of toxigenic staphylococci as an *in vivo* production of toxin by the bacteria.

It has been observed in this laboratory, that a strain of staphylococci which regularly produced a rapidly fatal bacteremia in mice after intraperitoneal inoculation proved to be relatively innocuous if given by the intravenous route. However, adrenalectomy reduced resistance to an intravenous challenge with this microorganism by a factor of at

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† The authors wish to acknowledge the technical assistance of Miss Martha Chamberlin in this study, and their indebtedness to Dr. Paul S. Nicholes for the use of the high frequency oscillator.

least 1000. This deficiency in resistance produced by adrenalectomy is apparently not caused by the impairment of phagocytic or cidal activities of the reticulo-endothelial system, but rather to an increased sensitivity of the host to the noxious effects of the bacterial protoplasm. Hydrocortisone effectively protected the adrenalectomized mice from the toxic effects of an intravenous challenge of either viable staphylococci or a bacterial extract.

Materials and methods. Mice: Eight-weeks-old male mice (CBA) were used throughout this study. Bilateral adrenalectomies were performed by the dorsal approach 18 hours before challenge. These animals were fed *ad libitum* and maintained on 0.9% saline as drinking water. All control mice were sham operated. In order to evaluate the biological effect of hydrocortisone, adrenalectomized mice were used to obviate interference from endogenous adrenal cortical secretion. *Bacteria*: The organism selected for this study was a strain of *Micrococcus pyogenes* var. *aureus*, which had been isolated from a human source. The bacterial cells were prepared for inoculation by growing them for 18 hours on nutrient agar, and then were transplanted to brain-heart infusion broth for an additional three hours of growth. The cells were harvested by centrifugation and washed once, and then resuspended with 0.9% saline. The concentration of bacteria was initially adjusted to a standard turbidity in the Klett-Summerson photocolorimeter, using a 420 mμ blue filter. This was later confirmed by plate counts. *Plate counts*: Determination of viable numbers of bacteria was routinely carried out by serial 10-fold dilution of the sample, of which 0.5 ml aliquots were taken for pour plating (nutrient agar). Three plates were used for each dilution per sample. The average count was obtained after the plates had incubated for 18 hours at 37°C. *Bacterial extract*: Bacteria were grown in brain-heart infusion broth for 18 hours then sedimented by centrifugation and washed 3 times with 0.85% saline. The washed preparation was sonerated in a Raytheon high frequency oscillator at 9.0 to 9.5 kilocycles for 4 hours at 13°C. The opalescent material was removed and centrifuged overnight in the cold. The supernatant was diluted to 1:5 and centrifuged 3 more times to reduce the viable cell count to less than 4/ml. The supernatant was then treated with $(NH_4)_2SO_4$ and the resultant precipitate dialyzed, reprecipitated with ethanol and finally washed with acetone and dried. *Treatment and challenge*: Hydrocortisone acetate (Merck)‡ was given by the intraperitoneal route in the standard volume of 0.1 ml. The hormone was prepared as a saline suspension and given in varying dilutions 2 hours prior to challenge. The challenge inocula of bacteria or bacterial extract were contained in volumes of 0.25 ml and introduced via the tail vein 18 hours after adrenalectomy. The LD_{90} (90% lethal dose = 86.9 ± 3.4%) of viable bacteria for adrenalectomized mice was found to be approximately 3 to 5 million viable cells, while the LD_{90} (90.2 ± 4.1%) dosage of the bacterial extract was found to consist of approximately 2.5 μg of the dried material.

Results. Experimental. Effect of route of infection on resulting bacteremia: In preliminary work it was found that the mouse was much more resistant to infection with this microorganism by the intravenous route than by the intraperitoneal. To investigate this further, mice were divided into 2 groups and injected with 5×10^8 viable bacteria contained in a volume of 0.25 ml. One group of animals received this challenge dose by the intravenous route, while the other group was inoculated intraperitoneally. At intervals of 5, 30, 60, 120, 180 and 240 minutes following injection, 3 mice from each group were lightly anesthetized and blood samples obtained by cardiac puncture with heparinized syringes. These samples were used for estimation of the existing bacteremia (plate counts). Mice not used for bleeding were kept as infected controls. All of the intraperitoneally infected controls were dead (8/8) 24 hours after injection, whereas none (0/8) of the intravenously injected controls exhibited any obvious symptoms of infection following injection with the

‡ The authors wish to thank Dr. Elmer Alpert of Merck and Co., Rahway, New Jersey for the generous supply of hydrocortisone used in these experiments.

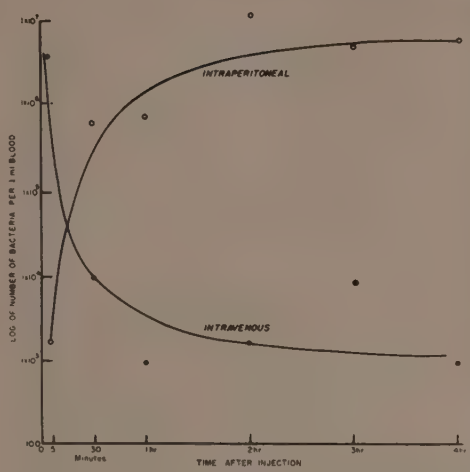

FIG. 1. Comparison of numbers of viable bacteria in blood of mice following infection by intravenous or intraperitoneal routes.

bacteria. It is evident (Fig. 1) that injection of the microorganism by the intraperitoneal route permitted the development of a rapid and overwhelming bacteremia which ultimately resulted in the death of the host. The initial bacteremia (5 minutes) undoubtedly was due only to invasion from the peritoneal cavity which was then maintained at this level by the constant infusion of bacteria produced by multiplication in the cavity. The actual extent of the invasion from the peritoneum cannot be fully appreciated unless the fate of the blood-borne bacteria is considered. The reticulo-endothelial system, which functions as a clearing mechanism for the blood, efficiently removed the inoculum dose of bacteria (Fig. 1), whereas the local response in the peritoneum is apparently overwhelmed allowing a fatal systemic invasion to occur. The two examples illustrate the extremes in bacteremia, which "represents a balance between bacterial multiplication, invasion of the blood stream, and the activity of the clearing mechanism"(10).

Bacteremia and splenic uptake of bacteria in adrenalectomized and sham operated mice: Adrenalectomy rendered the mouse highly sensitive to an intravenous inoculum of the bacteria that was relatively innocuous in the sham operated control. To investigate the influence of adrenal cortical secretion on this aspect of resistance, adrenalectomized and sham-operated mice were inoculated intravenously with approximately 3.3×10^6 bacteria. Blood samples were obtained by intracardial puncture at the intervals noted in Fig. 2. The blood samples for each separate period were obtained from at least five mice and pooled. Likewise, the spleens of these animals were rapidly and aseptically removed, weighed, pooled and homogenized with sterile 0.85% saline in glass TenBroeck tissue grinders. Aliquots of the blood and spleen homogenates were taken for plate counting.

The inoculum used would theoretically be sufficient to produce a bacteremia of approximately 10^6 cells per ml of blood, which indicates a precipitous loss of bacteria (Fig. 2) from the blood during the initial period following injection (ca. 90% in initial 5 minutes, ca. 90% of remainder during subsequent 25 minutes). The initial high count of viable bacteria in the spleen is well correlated with the rapid clearance of the bacteria from the blood. Supplemental studies have shown that the reticulo-endothelial activity of the liver (on weight basis) was similarly effective. Since the concentration of viable bacteria in the blood rapidly fell to a relatively insignificant level, the decrease in numbers of viable

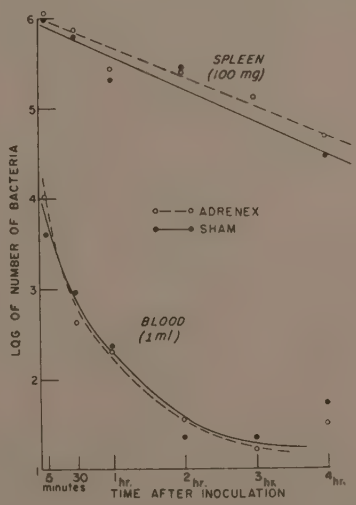

FIG. 2. Comparison of numbers of viable bacteria in blood and spleen of mice, adrenalectomized or sham-operated, following infection by intravenous route.

TABLE I. Independence of Lethal Dose on Numbers of Viable Staphylococci.

Bacterial preparation	No. viable bacteria per inoculum	Mortality	Mean survival time, min.	Probability*
A. Control	4.0×10^8	9/ 9	101.1	
Sonerated	6.3×10^6	10/10	85.9	P = .01
B. Control	2.0×10^8	10/10	112.6	
Sonerated	3.2×10^6	10/10	86.1	P = .01
Boiled	0.0×10^0	10/10	103.5	P = .3–.4

* Probability, using Student's "t" test, that the mean survival time of the test group is the same as the mean survival time of the control.

bacteria in the spleen was probably due to the cidal activity of the phagocytic cells. This decrease amounted to approximately 95% in 4 hours. All of the injected control sham-operated mice not used for bacteremia study survived with no evidence of symptoms, whereas all of the injected adrenalectomized mice succumbed. Despite the almost complete disappearance of bacteria from the blood and apparent destruction by the reticulo-endothelial system, symptoms of bacterial intoxication were evident in many of the adrenalectomized mice by the four hour period (ruffled fur, unsteady gait, etc.). The essential difference in the resistance of the adrenalectomized mouse when compared to its control seemed to be in its increased sensitivity to the bacteria *per se,* rather than a deficiency in ability to phagocytize and destroy the pathogen.

Toxic factor and resistance: From the results of the previous experiment it seemed possible that the digestive processes of the phagocytic cells of the reticulo-endothelial system might liberate toxic substances from the bacterial cell to the circulation which, in the adrenalectomized mouse, could attain a lethal concentration due to the greater sensitivity of this animal to noxious material. Preliminary observations showed that the lethality of various preparations of bacteria for adrenalectomized mice (measured as mean survival time) was more directly related to the concentration of bacterial substance than the numbers of viable bacteria in the inoculum. Bacteria formalinized and washed, heated to 58°C, exposed to boiling temperatures or incubated in distilled water or with antibiotics, were not less toxic than control bacterial suspensions. To simulate the result of digestive action by phagocytic cells on the ingested microorganisms, bacteria were "solubilized" by disruption in a high frequency oscillator (sonerated) for 4 hours at 13°C.

A comparison of the toxicity of sonerated and control preparations was made following intravenous injection of the materials into adrenalectomized mice. In part A of Table I it can be seen that "solubilization" of the bacteria significantly increased the toxicity of this preparation, even though the numbers of viable bacteria were markedly reduced. The experiment was repeated (B) using a 1 to 2 dilution of the control which had been immersed in a boiling water bath for 30 minutes. Analysis of the toxicity of the control vs. the heated preparation showed no significant difference, although the latter inoculum was completely sterilized by the heat treatment. On the other hand, "solubilization" of the bacteria significantly enhanced the toxicity of the preparation. The results suggest that since the intravenously injected bacteria are rapidly localized in the tissues of the reticulo-endothelial system and therein destroyed (Fig. 2), the lethal effect of the bacteria may depend on solubilization of the ingested bacteria by the phagocytic cells and subsequent release of the toxic material to the circulation of the sensitive host.

Protective effect of hydrocortisone: The observation has been made that staphylococci administered by the i.v. route are rapidly cleared from the blood and destroyed by the cells of the R. E. system. If the digestion of these phagocytized bacteria liberates toxic bacterial substance to the circulation and thereby fatally intoxicates the adrenalectomized mouse, replacement therapy should be effective in protecting mice against a lethal

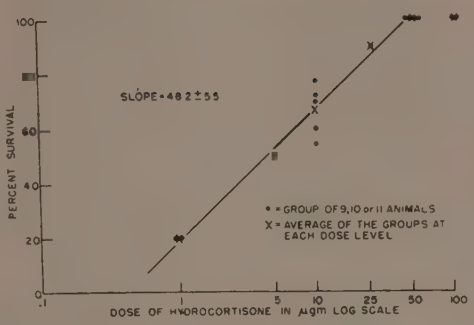

FIG. 3. Protective effect of hydrocortisone in adrenalectomized mice against 1 LD_{50} (i.v.) of a toxic bacterial extract.

dose of either soluble bacterial extract or viable bacteria. Adrenalectomized mice were treated with graded doses of hydrocortisone two hours prior to an i.v. injection of the cell-free bacterial extract. Observations on mouse survival were routinely carried out over a period of 72 hours.

Fig. 3 illustrates the various degrees of protection, relative to hormone dosage, provided to mice by hydrocortisone against a dose of bacterial extract that was lethal to 90.2 ± 4.1% of the untreated controls (40 mice). The straight line in Fig. 3 was obtained by the method of least squares. Hormone-treated mice surviving for 24 hours following the challenge died at a rate no greater than non-challenged adrenalectomized controls.

The animals used to test the effect of hydrocortisone in mice challenged with an i.v. dose of viable staphylococci were prepared, treated and observed in essentially the same manner as in the previous experiment. In the dosage range from 0.25 to 25 γ of

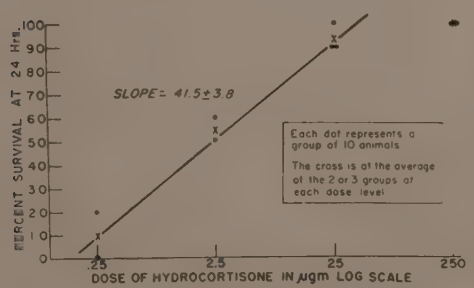

FIG. 4. Protective effect of hydrocortisone in adrenalectomized mice against 1 LD_{50} (i.v.) of viable *M. pyogenes* var. *aureus*.

hydrocortisone, a straight line relationship of log dose to response was obtained by the method of least squares (Fig. 4). It is interesting to note that a 10-fold increase in hormone dosage did not diminish the protective response (*i.e.*, 250 γ). Since non-moribund animals surviving 24 hours after injection died at a rate no greater than non-infected controls, it was evident that the hormone effect was not limited to a mere prolongation of survival time. Bacteremia in survivors, if present, was extremely light and the spleens contained few to no detectable organisms.

Discussion. The remarkable sensitivity of the adrenalectomized animal to noxious substances has provided a unique opportunity to study the pathogenesis of a controlled bacteremia. It has been found that after the introduction of bacteria into the blood of either adrenalectomized or sham-operated mice, the pathogens are rapidly removed from the circulation. Both the spleen and liver participated in the removal, and, on a weight basis, were similarly active. Following the uptake by the spleen, a sterilization of the bacteria ensued, and it is postulated that the digestion of the bacteria by the reticulo-endothelial cells resulted in a release of toxic (or irritating) bacterial substances. Harris and Ehrich (11) have reported that soluble antigens of locally injected washed Shigella bacteria or foreign erythrocytes appear at the site of injection, draining lymph node and in the efferent lymphatics, at times which suggest solubilization and release of these antigens from the phagocytic cells of the local area. It has been found that "solubilization" (by soneration) of the bacteria decreases the survival time of i.v. injected adrenalectomized animals even though the numbers of viable bacteria are greatly reduced. Inocula of bacteria sterilized by exposure to boiling water bath for 30 minutes were as toxic as viable control material.

Since the intact mouse is markedly resistant to a given dose of either the bacteria or the bacterial extract given by the intravenous route, it is likely that the release of soluble bacterial substances by the reticulo-endothelial system in the intact mouse is accomplished without any untoward effects. However, in

the adrenalectomized mouse, which is notoriously sensitive to bacterial toxins, etc., this destruction of the bacteria does not provide suitable protection. Perhaps in the intact mouse, which has been given an intraperitoneal inoculation of the bacteria, the resulting bacteremia (Fig. 1) assumes such proportions that the R. E. system accumulation of bacterial material produced during the period of infection may be sufficient to constitute a lethal dose within a relatively short time after injection (5 to 7 hours). Smith and Keppie (12) have shown the irreversible nature of bacterial intoxication with anthrax bacillus despite control of the bacteremia by antibiotics.

Single administrations of graded doses of hydrocortisone were shown to furnish protective responses relative to hormone dosage in adrenalectomized mice injected intravenously with an LD_{90} of either viable bacteria or bacterial extract. Since this protection did not deteriorate with time, the pathogenic activity of this organism after an intravenous injection must be essentially due to the release (solubilization) of its cellular components, rather than to multiplication and concomitant toxigenic activity. The apparent enhancement of the detoxification mechanisms of the sensitive host by hydrocortisone would require no new property not already generally ascribed to this hormone. The antiphlogogenic activity of hydrocortisone, by limiting the chain-like sequence of events due to a noxious stimulus, can so markedly reduce the systemic reaction to the stimulus as to completely inhibit the appearance of symptoms(4).

Summary. 1. The increased tolerance of mice to an i.v. challenge of staphylococci as compared to an injection of the bacteria by the i.p. route was found to be related to the rapid clearance of bacteria from the blood following the i.v. injection. However, adrenalectomy markedly reduced the resistance to an i.v. challenge without altering the rates of clearance or bacterial destruction by the R. E. system. 2. It was postulated that digestion of the bacteria by the R. E. system liberated bacterial material which was noxious to the adrenalectomized mouse. "Solubilization" of the bacterial preparation increased its toxicity as compared to control viable or heat-sterilized preparations, indicating the independence of this toxicity to numbers of viable bacteria. 3. Hydrocortisone pretreatment of adrenalectomized mice was found to be protective, relevant to dosage, in minute amounts, against i.v. challenge with either viable staphylococci or extracts of this organism.

1. Perla, D., and Marmorsten, J., 1941. *Natural Resistance and Clinical Medicine,* Little, Brown and Co., Boston, p475.

2. Keefer, C. S., 1954, in Lukens, F. D. W., *Medical Uses of Cortisone Including Hydrocortisone and Corticotropin.* The Blakiston Co., New York, p401.

3. Kass, E. H., and Finland, M., *Ann. Rev. Microb.,* 1953, v7, 361.

4. Dougherty, Thomas F., *Progress in Allergy,* 1954, v4, 319.

5. ———, *Adrenal Cortex,* Tr. 2nd Conf. Josiah Macy, Jr. Foundation, New York, 1950, p88.

6. Robinson, H. J., Mason, R. C., and Smith, A. L., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 312.

7. Hill, D. W., Brown, H. E., and Gebhardt, L. P., *Fed. Proc.,* 1954, v13, 431.

8. Germuth, F. G., Ottinger, B., and Oyama, J., *Bull. Johns Hopkins Hosp.,* 1952, v91, 22.

9. Kleiger, B., Blair, J. E., and Hallman, F. A., *Arch. Surg.,* 1942, v45, 571.

10. Bennett, J. L., and Beeson, P. B., *Yale J. Biol. Med.,* 1954, v26, 241.

11. Harris, T. N., and Ehrich, W. E., *J. Exp. Med*, 1946, v84, 157.

12. Smith, H., and Keppie, J., *Nature,* 1954, v173, 869.

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Bacteriostatic Properties of Some Derivatives of DDT.*† (22000)

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During an investigation of the fate of DDT in resistant insects, a number of related compounds were synthesized. Since a survey of the literature disclosed little information concerning the effects of these compounds on the metabolism of microorganisms, they were also screened for possible bacteriostatic properties.

The compounds are in general quite water insoluble and it was very difficult to obtain reproducible results using the usual turbidimetric methods to estimate microbiological growth. Such methods are also laborious, time consuming and require large amounts of compound when testing over a wide range. For these reasons the gradient plate technique of Szybalski(16-18) was utilized for quick approximate screening.

Materials and methods. To a slanted petri plate 9 cm in diameter was added 20 ml of sterile nutrient agar (Difco) so that the bottom was just covered. After solidification the plate was leveled and an additional 20 ml of agar containing the appropriate concentration of compound to be tested was added. A waiting period of from 2 to 5 hours at 25°C proved to be sufficient to allow the compound to diffuse vertically and establish a uniform concentration slope by virtue of the thickness

TABLE I. Concentration of DDT Derivatives Required to Inhibit Growth of Microorganisms.

Compound	Source	Microorganism* 1	2	3	4	5
Benzophenone	†					
4,4′-Dichlorobenzophenone	13					
4,4′-Dimethoxybenzophenone	†					
4,4′-Dichloro-3,3′-dinitrobenzophenone	1					
4,4′-Dichloro-3,3′-diaminobenzophenone	12					
4-Chlorophenyl 2-thienyl ketone	4					
Phenyl 2-thienyl ketone	3				54‡	
4,4′-Dimethylaminobenzophenone	†					
4-Fluoro-3-methylbenzophenone	10					
Benzhydrol	2	14	112			
4,4′-Dichlorobenzhydrol	11		112	140	42	
4,4′-Dimethoxybenzhydrol	14					
4,4′-Dichloro-3,3′-dinitrobenzhydrol	10	14	70		14	14
4,4′-Dichloro-3,3′-diaminobenzhydrol	10					
4-Chlorophenyl-2-thienylmethanol	10	70	112	70	42	42
Phenyl-2-thienylmethanol	9		132			
4,4′-Dimethylaminobenzhydrol	8				42	14
α-Methylbenzhydrol	2					
1,1-bis(p-Chlorophenyl) ethanol	5		42	42	42	
1,1-bis(p-Methoxyphenyl) ethanol	6					
1-Phenyl-1(2-thienyl) ethanol	10	224	70			
1-(4-Fluoro-3-methylphenyl)-1-phenylethanol	10		42	14	14	
1,1-bis(4-Chloro-3,5-dinitrophenyl) ethane	7		112	14	14	112
bis(p-Chlorophenyl) acetic acid	15				14	14
4-Fluoro-3-methylbenzhydrol	10	14	14		14	14

* (1) *E. coli* (sucrose negative); (2) *M. pyogenes* HSR9674; (3) *S. faecalis* ATTC1170; (4) *S. ellipsoideus*; (5) *A. suboxydans* ATTC621.

† Eastman Kodak Co.

‡ Values are given in μg/ml required to inhibit growth. Where no value is given it is taken to mean that growth of the microorganism was not affected by the highest concentration used.

* Technical Paper No. 930, Oregon Agric. Exp. Station.

† Data taken from a thesis submitted in partial fulfillment of requirements for the Ph.D., Department of Chemistry, Oregon State College, June 1953.

‡ Present address: Western Utilization Research Branch, Albany, Calif.

ratio of the agar layers. The plates were then inoculated with a thin streak of organisms using a small sterile brush. The plates were inverted and incubated for 18 hours. At this time the growth of the organisms could easily be seen and the point where growth was just inhibited determined. Concentrations up to 250 μg per ml were run at least in duplicate. The microorganisms were obtained from stock cultures belonging to the Department of Bacteriology, Oregon State College. The values obtained by this method compared within 15% of those found by standard dilution techniques when using phenol or penicillin G, sodium salt.

Results. In Table I is summarized the results obtained by screening the miscellaneous compounds for their abilities to inhibit growth of the five selected microorganisms. In general the ketones were inactive at a concentration of 250 μg per ml which was the highest level used. Many of the alcohols, however, were quite active in inhibiting growth, some at rather low concentrations. In addition to the organisms listed, most of the compounds prepared were screened against *Candida albicans*. In general no inhibitory effects were noted against this pathogenic yeast; however, 4, 4′-dichlorobenzhydrol completely inhibited growth at 140 micrograms per ml.

Summary. A number of miscellaneous compounds related to DDT have been screened for their abilities to inhibit the growth of 5 select bacteria and yeasts. The alcohols were much more active than the corresponding ketones.

1. Backeberg, O. O., and Marais, J. L. C., *J. Chem. Soc.*, 1945, v1945, 803.
2. *Org. Synth. Col.*, 1941, v1, 90.
3. ———, 1943, v2, 520.
4. Buu-Hoi, Ng. Ph., Ng. Hoan, and Ng. D. Kuong, *Redueil des travaux chimiques des pays-bas*, 1950, v69, 1085.
5. Grummitt, Oliver, Buck, Allen H. and Becker, E. I., *J. Am. Chem. Soc.*, 1945, v67, 2265.
6. Grummitt, O., and Marsh, D., *ibid.*, 1948, v70, 1289.
7. Forrest, James, Stephanson, O., and Waters, W. A., *J. Chem. Soc.*, 1946, v1946, 333.
8. Mastagli, P., *Compt. Rendus.*, 1937, v204, 1656.
9. Minnis, W., *J. Am. Chem. Soc.*, 1929, v51, 2143.
10. Moore, J. E., Ph.D. Thesis, Oregon State College, 1953.
11. Montagne, P. J., *Recueil des travaux*, 1905, v24, 105.
12. ———, *Berichte*, 1915, v48, 1027.
13. Newton, H. P., and Groggins, P. H., *Ind. and Eng. Chem.*, 1935, v27, 1307.
14. Schnackenberg, H., and Scholl, R., *Berichte*, 1903, v36, 654.
15. Smith, M. I., *J. Pharm. and Exp. Ther.*, 1946, v68, 359.
16. Szybalski, W., *Science*, 1952, v116, 46.
17. ———, *Bact. Proc.*, 1952, v1952, 36.
18. Szybalski, W., and Bryson, V., *J. Bact.*, 1952, v64, 489.

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Failure of Y^{90} to Escape from Skeletally-Fixed Sr^{90}.* (22001)

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In the evaluation of the toxicity of radioactive elements deposited in living animals it is essential to know how much energy is delivered to the irradiated tissues. When the radioactive element gives rise to daughter products of different atomic species possessing physical and chemical properties different from the parent species, particular caution is necessary. This fact was recognized early in the work on radium toxicity when it was discovered that a large fraction of Rn^{222} escaped from skeletally deposited Ra^{226} and was exhaled in the breath. It has also been found recently(1) that considerable ThX (Ra^{224})

* Supported by U. S. Atomic Energy Commission.

FIG. 1. a. Autoradiogram of slab of bone taken from proximal end of right humerus (magnification 1.5×). b. X-ray picture of same slab of bone at same magnification.

daughter escapes from parent RdTh (Th^{228}) deposits in bone. Sr^{90} is an important component of the radioactive fallout from atomic explosions. In considering its toxicity, particular interest is focused on the contribution to beta ray dosage which is made by the more energetic beta rays of its 2.7 day daughter, Y^{90}. A comparison of the energies of the Sr^{90} and Y^{90} beta rays shows that, under conditions of radioactive equilibrium, tissue would receive approximately four times as much energy from the more energetic beta rays of Y^{90}. Hence, we have studied the metabolic fate of the potentially more damaging Y^{90} formed from Sr^{90} deposits in the skeleton of a young beagle dog. The following are the possible metabolic pathways for the Y^{90}: (1) Excretion from the body; (2) Retention at sites of formation in the skeleton; (3) Escape from sites of formation and re-deposition in skeleton in different sites; (4) Some combination of the above.

Our experiments show that most or all of the Y^{90} daughter is retained at the sites of formation in the skeleton.

Methods. Our experiments were carried out on a single beagle dog which was sacrificed 4 months following the intravenous administration of 150 μc per kg of Sr^{90} at equilibrium with its Y^{90} daughter. The injection solution consisted of Sr^{90}, Y^{90} in a citric acid, sodium citrate buffer of pH = 3.5 and 0.08 M total citrate. The dog was 5 months old at the time of injection, and therefore, its skeleton was growing during the presacrifice interval. The fate of the Y^{90} formed from Sr^{90} decay in the bone was studied in 3 ways. 1. The right humerus was cut longitudinally into slices 1-2 mm thick, embedded in Gelva resin,† and cemented on aluminum plates. After grinding the slabs flat with fine abrasive paper, contact autoradiograms were made on Kodak no-screen x-ray film. Fig. 1a is an autoradiogram (magnified 1.5x) of one of these slabs from the proximal end of the humerus; Fig. 1b is an x-ray picture taken at the same magnification. Another slab was cut along the parallel lines shown in Fig. 1a; adjacent lines are about 1 mm apart. The bone of section X was being deposited at the time of Sr^{90} injection and the bone of sections X + 1, X + 2, etc. was deposited at progressively later times. Following wet ashing, these samples, and also samples of bone from the shaft, were counted in a

† Shawinigan Products Corp., N. Y. City.

Geiger counter. Measurements were made both with and without an aluminum absorber sufficiently thick (0.7 mm) to absorb all Sr^{90}, but not all Y^{90} beta rays. A series of such counts was made from 6 hours to 7 days after sacrifice. In all samples, the Y^{90} beta ray activity was at equilibrium ($\pm$ 3%) with the parent Sr^{90} at 6 hours after sacrifice.

Results. If Y^{90} produced *in vivo* escaped from its site of formation into the general circulation, one would anticipate its concentration in the primary spongiosa beneath the epiphyseal line, in a manner similar to the deposition of parenterally administered Y^{90}(2). The amount of Sr^{90} in the primary spongiosa is quite low since this was a growing dog and the bone of this area was deposited at a considerable time after injection of Sr^{90}. Because of this low Sr^{90} concentration, an excess of Y^{90} could have been readily detected. The fact that no such excess of Y^{90} occurred in the primary spongiosa indicates that little, if any, of the Y^{90} produced *in vivo* escaped from its sites of formation.

Fig. 1b also clearly demonstrates that metaphyseal trabecular bone containing a high concentration of radioactivity (section X) is markedly opaque to x-rays. This opacity is due to the presence of bone of abnormally high density in this region and is evidence of radiation damage. This point will be discussed further in a future publication. A similar effect can be seen in the radius of this dog (Fig. 2).

II. The left radius was divided into 8 segments as illustrated in the x-ray picture (Fig. 2). The pieces were quickly dissolved in nitric acid and aliquots were mounted for beta ray measurement. Aliquots of the samples were measured from 8 hours to one week after sacrifice by 2 methods. The Sr^{90} and Y^{90} beta rays were measured in a 2-π proportional counter; Y^{90} only was measured using an end-window G-M tube with 0.7 mm aluminum between sample and detector. Similar measurements were made on the heads and shafts of the metacarpals. The data show no significant variation with time. The measurements of Sr^{90} and Y^{90} beta rays with time are rather insensitive, and the conclusion from these data is that the Y^{90} activity at the time of sacrifice

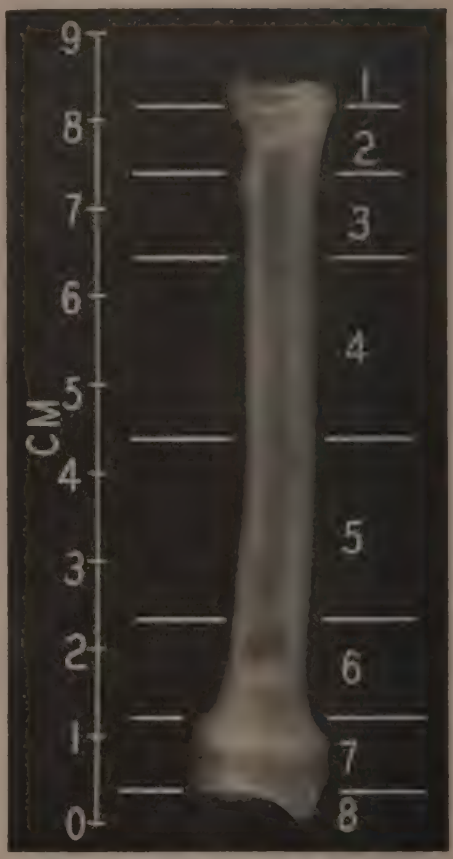

FIG. 2. X-ray picture of left radius.

equals the Sr^{90} activity ($\pm$ 20%). The Y^{90} beta ray measurements are more sensitive, and show that the Y^{90} activity at the time of sacrifice equals the Sr^{90} activity ($\pm$ 10%). Hence, Y^{90} is close to equilibrium in these bone samples which differ both in Sr^{90} concentration and morphologic composition.

Measurements were also made on the liver and plasma of this dog. The liver was homogenized, and an aliquot of the homogenate was wet ashed, as was a plasma sample. Aliquots of these samples were plated, and total beta ray activity was measured as described above.

In the plasma, Y^{90} was not in equilibrium with its parent; the Y^{90} activity was approximately half the Sr^{90} activity. In the liver, Y^{90} was in equilibrium, and the liver concentration

of Sr^{90} was about equal to the plasma Sr^{90} concentration. Since the skeleton contains approximately 5000 times as much Sr^{90} as the liver, measurement of the liver for excess Y^{90} is sufficiently sensitive to detect the escape of a small fraction of the Y^{90} formed in the skeleton, even though the deposition in the liver of parenterally administered yttrium is low. If it is assumed that any Y^{90} which leaves its site of formation in the skeleton behaves as parenterally administered tracer yttrium(3), then the lack of excess Y^{90} in the liver shows that there is no appreciable escape from the skeleton of *in vivo* formed Y^{90}.

III. The x-rays produced by the Y^{90} beta rays traversing the bony tissue of the right radius were measured as a function of time after sacrifice and of position along the axis of the bone. X-ray production by a continuous spectrum of beta rays of maximum energy E_{max} striking a thick target is approximately proportional to E^2_{max}(4). Hence the yttrium x-ray production is approximately $(2.24/0.54)^2 = 17$ times as intense as the strontium x-ray production and is responsible for most of the counter response.

A ¼ inch separation between lead bricks was used to collimate the x-rays; the detector was a NaI(Tl) scintillation counter. Sufficient thickness of lucite was used to absorb all beta rays. A series of measurements at ¼ inch intervals along the length of the right radius was made. These measurements showed no growth or decay of x-ray production (to $\pm$ 5%) along the length of the bone. Since the x-rays detected were almost entirely generated by Y^{90} beta rays, these measurements show the presence of Y^{90} rather than Sr^{90}. Hence, the Sr^{90} in all segments of the right radius was close to equilibrium with the *in vivo* formed Y^{90}.

Summary. The data presented demonstrate that Y^{90} produced *in vivo* from long term skeletal deposits of Sr^{90} in young dogs does not escape from the local areas of bone in which it is produced. This is in agreement with previous experimental evidence(5). The implications of this finding are as follows: First, the combined energy of the Sr^{90} and Y^{90} beta rays irradiates the skeleton. Hence, dosimetric data determined *in vitro* can be directly applied to *in vivo* conditions. Second, the Sr^{90} deposited in the growing skeleton is probably intracrystalline. This is in agreement with the x-ray diffraction findings of McDonald(6). Work is in progress to see whether this effect occurs in older dogs where there is less bone growth and less crystal formation.

1. Van Dilla, M. A., Stover, B. J., and Arnold, J. S., *Am. J. Roentgenol.*, in press.

2. Hamilton, J. G., *Rev. Mod. Phys.*, 1948, v20, 718.

3. Schubert, Jack, *et al.*, *J. Biol. Chem.*, 1950, v182, 635.

4. Introduction to the Atomic Nucleus, Robley D. Evans; MIT course notes to be published, Fall, 1955 by McGraw-Hill (N.Y.).

5. *Biological Hazards of Atomic Energy*, Ed. by A. Haddow, p. 152-153. Oxford Press, 1952.

6. McDonald, N., Ezmirliam, F., Spain, P., and McArthur, C., *J. Biol. Chem.*, 1951, v189, 387.

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Occurrence and Activation of an Elastase Precursor in Pancreas. (22002)

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Lansing and coworkers(1) have found that in teleost fish elastase occurs in pancreatic islet tissue but not acinar tissue. They cited, among reasons for studying this question, the findings that an herbivore (bovine) pancreas produces elastase and that the enzyme has not been detectable in human pancreatic juice. Kokas and coworkers(2) reported the presence of elastase in dog pancreatic juice, but occasionally it could not be found even when proteolytic activity could be measured.

Obviously, the establishment of an islet origin for elastase would indicate an endocrine function. Before an acinar origin and an occurrence in pancreatic juice could be unequivocally excluded, the possibility of an activatable zymogen was explored. The present study indicates that in dog pancreatic juice and in hog pancreas extracts the elastase does exist in the zymogen form, and a duodenal factor or trypsin is capable of converting this precursor to active elastase.

Methods and materials. Activations were carried out at 24°C for 20 minutes with no shaking. Each system contained 0.4 ml of dog pancreatic juice or hog pancreatic extract in a total of 1.4 ml. Other components and their concentrations per tube were: crystalline trypsin and crystalline α-chymotrypsin, 0.2 mg; crystalline soybean trypsin inhibitor, prepared according to Kunitz(3), 2 mg; amorphous pancreatic trypsin inhibitor, prepared according to Kazal, *et al.*(4), 2 mg; 0.4 ml of a 20% aqueous extract of the dried-defatted hog duodenum; and 0.02M Sorensen phosphate buffer, pH 7.4. The juice, which was kindly supplied by Dr. H. Necheles of Michael Reese Hospital, was collected by cannulation after the dog was treated with 100 units secretin, 3 mg mecholyl, and nembutal. The extract was a clarified 2% solution of a lyophilized 25% aqueous extract (pH 7.0) of fresh hog pancreas. The assay system was a modification of that of Balo and Banga(5). Elastin was prepared from beef aorta after removal of collagen with boiling 1 N NaOH. The tubes contained 20 mg elastin, 0.5 ml of 0.5 M Sorensen phosphate buffer pH 7.4, 1.0 ml enzyme (activation mixture), and H_2O to 5.0 ml. Assays were performed for 30 minutes at 37°C with shaking. The tubes were chilled to 1°C, and their contents were filtered (S and S 595 paper). Total protein was determined in the filtrate by a Folin phenol method(6) in which crystalline bovine albumin was used as the standard.

Results. The activation studies are shown in Table I. Elastase is secreted in pancreatic juice in an inactive state capable of being converted to the active enzyme by trypsin or a duodenal factor but not by α-chymotrypsin. The duodenal effect is abolished by soybean and pancreatic trypsin inhibitors if the inhibitors are present from the beginning of the activation period. If present only during the assay they do not completely block the activated elastase. In control experiments, these inhibitors very effectively blocked the hydrolysis of denatured hemoglobin by trypsin; with an inhibitor to enzyme weight ratio of 1, the percent inhibitions by the soybean and pancreatic inhibitors were 87 and 80, respectively.

Discussion. The foregoing data point to an

TABLE I. Activation of Pro-elastase.

Activator	Elastase activity* Dog pancreatic juice	Elastase activity* Hog pancreatic extract
None	.7	.9
Trypsin	6.3	4.3
α-Chymotrypsin	.9	.0
Duodenal extract	5.8	3.7
Duodenal extr. + soybean trypsin inhibitor	.8	.4
Idem†	1.9	2.8
Duodenal extr. + pancreatic trypsin inhibitor	.6	.5
Idem†	1.3	2.6

* mg net solubilized protein.

† Inhibitors were added to activation tubes immediately before the aliquot was withdrawn for assay.

indirect role for duodenum—one of activating trypsinogen. Nascent trypsin appears to be the direct activating agent for pro-elastase. The absence of elastase activity in trypsin has been shown by Balo and Banga(5), and we have found it to be absent in both trypsin and α-chymotrypsin.

Several investigators have reported active elastase in extracts of desiccated pancreas without a duodenum component(5,7,8). These observations might be reconciled in the light of varying amounts of active protease in fresh mammalian pancreas tissue(9). Unless the starting tissue has an exceedingly low concentration of active trypsin or the investigator has taken special steps to avoid autoactivation, a given pancreas extract may be expected to contain sufficient trypsin to activate pro-elastase.

The data shown in Table I suggest that the duodenal factor is enterokinase. The activation mechanism strongly resembles that of chymotrypsinogen(10) and pro-carboxypeptidase(11) and may be represented as

(A) Trypsinogen $\xrightarrow{\text{enterokinase}}$ trypsin

(B) Pro-elastase $\xrightarrow{\text{trypsin}}$ elastase

Summary. Dog pancreatic juice and extracts of hog pancreas contain elastase in an inactive form. Trypsin or a duodenal factor is capable of activating pro-elastase. The activation is blocked by pancreatic and soybean trypsin inhibitors, an indication that the duodenal factor is enterokinase.

1. Lansing, A. I., Rosenthal, T. B., and Alex, M., Proc. Soc. Exp. Biol. and Med., 1953, v84, 689.

2. Kokas, E., Foldes, I., and Banga, I., *Acta Physiol. Acad. Sci. Hung.*, 1951, v2, 333.

3. Kunitz, M., *J. Gen. Physiol.*, 1946, v29, 149.

4. Kazal, L. A., Spicer, D. S., and Brahinsky, R. A., *J. Am. Chem. Soc.*, 1948, v70, 3034.

5. Balo, J., and Banga, I., *Biochem. J.*, 1950, v46, 384.

6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

7. Banga, I., *Acta Physiol. Acad. Sci. Hung.*, 1952, v3, 317.

8. Pepler, W. J., and Brandt, F. A., *Brit. J. Exp. Path.*, 1954, v35, 41.

9. Robbins, K. C., Grant, N. H., and Schlueter, R. J., unpublished data.

10. Northrop, J. H., Kunitz, M., and Herriot, R. M., *Crystalline Enzymes*, Columbia Univ. Press, New York, 1948, 96.

11. Anson, M. L., *J. Gen. Physiol.*, 1937, v20, 777.

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Clostridium perfringens Iota Antitoxin Levels In Convalescent Sera from Hemorrhagic Fever Patients. (22003)

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The resemblance of the anatomic-morphologic changes in men who died of hemorrhagic fever (HF) to those in animals which succumbed to enterotoxemias caused by *Clostridium perfringens* led to an inquiry into the possible role of *Cl. perfringens* in the Korean variety of that human disease(1-4). The occurrence of *Cl. perfringens* in the human intestine is well known. Past studies concerned with the distribution of toxigenic types of *Cl. perfringens* have demonstrated that types A and F constitute all of the human intestinal Isolates with a few exceptions(5-8). The classification of the strains isolated from the colon of HF patients has not yet been undertaken due to the present limited supply of typing antitoxin on hand. It seemed, however, that the demonstration of serum antitoxins against known species of *Cl. perfringens* in convalescent HF patients would be another approach to this problem.

Materials and methods. Sera. Sera were obtained from 17 patients on the Hemorrhagic Fever Wards of an Army Hospital in Korea, during their convalescence from a disease answering the clinical criteria of HF. These

sera were drawn 35 to 51 days after onset of the disease and were compared with sera of 50 individuals from Japan and areas other than Korea who had never suffered such an illness. *Toxins.* Crude toxins were prepared from the following known type strains of *Cl. perfringens*: type A, No. 146 from Dr. L. S. McClung, Univ. of Indiana; type B, No. CN 667; type C. No. CN 882; type D, No. CN 1635; type E, No. CN 1241, and type F, No. CN 2048 from Dr. H. Warrack of the Wellcome Laboratory, Beckenham, England. The *Cl. perfringens* strains were grown in a horse muscle infusion containing 1% papain digest of horse muscle and 30% horse muscle particles, as recommended by Ross(9). The cultures were incubated for 18 hours at 35°C, centrifuged at high speed for 15 minutes to remove the large particles and sterilized by Seitz filtration. A later batch of type E toxin was sterilized by passage through a millipore filter with less loss of toxigenic material. All filtrates, except type A, were neutralized with alpha antitoxin to eliminate that toxin which is common to all recognized types of *Cl. perfringens*. The epsilon toxin of type D was activated with trypsin prior to use, as recommended by Batty and Glenny(10). This activation was not applied for the iota toxin of type E, as no enhancing value was noted after 7 hours incubation(9). The filtrates were tested against known antitoxins to confirm their type.* The L+ of each toxin was determined by the Ross method(9). *Procedure with HF sera.* Screening tests were performed by adding 0.6 ml sera which had been inactivated at 56°C for 30 minutes to 1.8 ml toxin containing 6 L+ mouse units, incubating the mixture for 30 minutes at room temperature and inoculating 0.5 ml of this mixture intravenously into each of 4 mice weighing 17-20 g. Neutralization was considered to occur if the mice survived for 72 hours. Quantitative determinations were performed as follows: The sera were diluted 1:2, 1:4, 1:6, 1:8 and 1:10 with physiological saline. One ml of each dilution was mixed with 1.2 ml toxin containing 6 L+ units and tested as in the screening test. Control sera were tested in the same manner using undiluted and 1:2 diluted sera. All titrations were repeated on 3 different days to confirm the reproducibility of results.

* *Cl. welchii* antitoxins supplied by Burroughs Wellcome and Company.

TABLE I. Individual Iota Antitoxin Levels of 67 Sera.

Units/cc serum	No. sera showing antitoxin	
	HF sera	Control
0 - .3	1	27
.4- .9	0	14
1 - 2.9	1	9
3 - 4.9	0	0
5 - 6.9	8	0
7 - 8.9	4	0
9 -11.9	2	0
12 -21	1	0
Total	17	50

Results. In the preliminary tests no neutralization of toxins produced by *Cl. perfringens* types A, B, C, D, or F was observed. Sera from 15 of the 17 HF convalescents contained sufficient antibody to neutralize the iota toxin produced by *Cl. perfringens* type E (Bosworth). Table I indicates the antitoxin titers of these 17 test sera as well as of the 50 control sera.

Sera tested against type E filtrates not neutralized with alpha antitoxin failed to protect the mice. Death was accompanied by hemoglobinuria which is common in lecithinase intoxication. This was not observed in mice killed with type E filtrate in which the alpha toxin was neutralized.

Discussion. The only major toxin produced by *Cl. perfringens* type E not formed by the other toxigenic strains is the iota toxin. Iota toxin is lethal, necrotizing, non-hemolytic and is activated by trypsin.

It is assumed that the neutralization observed was due to the presence of iota antitoxin as antitoxins of the other toxigenic types failed to protect mice when inoculated with type E filtrates used in these tests. The occurrence of iota neutralizing antibodies in the sera of convalescent HF patients in higher concentrations than in the 50 control sera is of interest. Further studies are being carried out to determine if an increase in iota antitoxin occurs during the course of HF. Cultural studies are also being conducted to determine the toxigenic types of *Cl. perfringens*

isolated from the stools of HF patients and from the soil of the endemic area in Korea.

Summary. Sera from 17 patients convalescing from hemorrhagic fever and 50 normal controls were tested for the presence of neutralizing antibodies of *Cl. perfringens* iota toxin. Hemorrhagic fever sera contained a significantly higher quantity of neutralizing antibodies than did the control sera.

We should like to express our thanks to Dr. McClung for the culture of *Cl. perfringens* type A, and to Dr. Warrack for the other toxigenic types, as well as for the type antitoxins made available by her from the Wellcome Research Laboratory.

1. Lukes, R. J., *Am. J. Med.*, 1954, v16, 639.
2. Bosworth, T. J., *J. Comp. Path.*, 1943, v53, 245.
3. Baldwin, E. M., Frederick, L. D., and Ray, J. D., *Am. J. Vet. Res.*, 1948, v9, 296.
4. Oakley, C. L., *Bull. Hyg.*, 1943, v18, 781.
5. Taylor, A. W., and Gordon, W. S., *J. Path. Bact.*, 1940, v50, 271.
6. Brothwick, G. R., *Brit. J. Exp. Med.*, 1937, v18, 475.
7. Taylor, A. W., and Gordon, W. S., *J. Path. Bact.*, 1940, v50, 271.
8. Hain, E., *Brit. Med. J.*, 1949, v1, 271.
9. Ross, H. E., Warren, M. E., and Barnes, J. M., *J. Gen. Microbiol.*, 1949, v3, 148.
10. Batty, I., and Glenny, A. T., *Brit. J. Exp. Path.*, 1947, v28, 110.

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Improved Procedure for Hypophysectomy of the Mouse.* (22004)

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As early as 1933, Selye, Collip and Thomson(1) reported the successful removal of the pituitary from female albino mice; no data on survival were included, although it was stated that mice hypophysectomized by the parapharyngeal approach tolerate the operation better than do rats. Four years later, Leblond and Nelson(2) published an extensive report on the histological changes observed in the adrenal, thyroid, and gonads of mice of the A and CBAN strains following hypophysectomy; a high mortality during the first postoperative week was reported, although the animals that survived this critical period subsisted thereafter apparently with no special therapy. Subsequently, Thomas(3) described a technic for which he used 2 minute hooks in place of the more customary dental drill to break through the cranium. The procedure for mouse hypophysectomy described in the present communication utilizes the basic technic of Smith(4), but introduces modifications which insure completeness of operation and make possible an operative survival approaching 100%.

Materials and methods. Materials. A cork-topped board of convenient size is outfitted with 2 rubber bands held taut by thumbtacks in order to secure the limbs. For immobilizing the head, a malleable wire is stuck firmly into the board and bent so that the incisors of the animal can be hooked securely beneath it. A 20-gauge one-inch injection needle bent at an angle of 120° serves admirably as a tracheal cannula. Cotton pledgets or miniature swabs are made by winding a small piece of cotton around the end of a pointed toothpick. These materials, together with a view of the operative setup, may be seen in Fig. 1. Other necessary equipment, forceps, dental drill, No. 5 dental burr, binocular magnifier mounted on a stand, and adjustable lighting, are similar to those required for the operation in the rat (5). Nembutal is used for anesthesia. 850 mg of sodium pentobarbital (USP) dissolved in 4 ml of propylene glycol, 2 ml of ethanol, and 14 ml of water serves as a stable stock

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FIG. 1. Operative setup for hypophysectomy of the mouse.

solution.† After the concentrated solution is diluted with distilled H_2O (1:4), injections are administered in a dose of 0.01 ml (0.085 mg of sodium pentobarbital) per g of body weight. *Operative technic.* Just before operation, the mouse is injected intraperitoneally first with the anesthetic agent and then with 0.25 mg of cortisone. The ventral neck region is shaved, a longitudinal midline incision, measuring 1 cm, is made, extending downward from the center of the lower jaw, and the sternohyoid muscle is separated so that the curved injection needle can be hooked gently into the trachea. Following cannulation, the base of the skull is reached by the usual parapharyngeal approach, as described for the rat(5). In the mouse, special care must be taken to distinguish between the synchondrosis itself and the anteriorly located transverse venous sinus. For the best exposure of the underlying pituitary, the hole drilled into the cranium should be so placed that one-third is above and two-thirds are below the occipito-sphenoidal synchondrosis. The intact lobes are then gently aspirated from the hypophyseal fossa; strong suction must be avoided since it ruptures the protective membrane which separates the pituitary from the brain proper. Upon removal of the gland, a pledget is inserted into the hole and the skin is closed with a single suture. Bleeding is usually negligible, and the pledget is removed before the animal is resuscitated. *Postoperative care.* Immediately following operation, the animal is placed in an atmosphere of 95% O_2-5% CO_2 until it recovers. It is then injected intraperitoneally with 1 mg of terramycin dissolved in 1 ml of 5% glucose solution. A similar injection is given on the second postoperative day along with a subcutaneous injection of 0.25 mg of hydrocortisone. Most strains require no additional therapy. For the best assurance of survival, hypophysectomized mice should be kept in a room free from drafts, at a constant temperature of approximately 82° F. *Completeness of hypophysectomy.* Immature female mice of the BALB/c, A/He, C_{57}, C3H and Swiss albino strains were hypophysectomized at 35 days of age by the technic described above. One month later they were sacrificed and their adrenals, ovaries and uteri were examined by gross observation for evidence of atrophy. These organs were then removed, dissected free from surrounding tissue and weighed on a Roller-Smith torsion balance. The non-operated animals were treated in a similar manner.

The *results* are summarized in Table I. At the time of autopsy, the hypophyseal fossa was also examined with a binocular microscope for possible pituitary remnants. Serial histological sections of the region were not made, since nearly all the operated animals showed marked atrophy of the organs associated with hypophyseal function.

Discussion. The method outlined herein is a modification of the technic developed by Smith(4) for hypophysectomy of the rat. It has been used with success on 5 strains of mice whose body weight is not less than 12 g. In smaller animals, difficulties in cannulation, and bleeding from the cranium, have been encountered. Some workers prefer to dispense with tracheal cannulation and to

† The commercial solution of nembutal was found to be unsatisfactory.

TABLE I. Effects of Hypophysectomy on Female Mice Operated on at 35 Days.

Strain	Operative condition*	No. of animals	Body wt, g: Day of operation	Body wt, g: At autopsy	Organ wt, mg: Adrenal	Organ wt, mg: Ovary	Organ wt, mg: Uterus	Width of tibial epiphyseal cartilage plate, μ
BALB/c	N†	10	15	19	5.4 ± .2‡	5.1 ± .04	45.7 ± 9.3	103.5 ± 2.2
	H	8	16	13	2.1 ± .2	1.1 ± .07	5.9 ± .6	71.2 ± 1.5
A/He	N	11	15	20	3.9 ± .2	4.7 ± .3	51.5 ±28.4	120.8 ± 5.9
	H	10	16	13	1.5 ± .1	1.1 ± .06	5.3 ± .7	60.2 ± 1.1
C57	N	10	13	19	3.9 ± .2	6.4 ± .3	65.5 ± 3.2	119.2 ± 1.7
	H	8	15	14	1.5 ± .1	1.1 ± .1	4.9 ± .5	48.5 ± 2.3
C3H	N	7	18	23	4.0 ± .2	10.1 ± .2	85.1 ± 9.1	119.8 ± 2.8
	H	10	18	15	1.6 ± .2	1.2 ± .1	6.1 ± .6	58.0 ± 1.1
Swiss albino	N	4	19	23	8.7 ± .01	7.0 ±1.6	50.1 ± 8.7	123.2 ± 2.6
	H	5	22	19	2.7 ± .4	2.2 ± .3	10.2 ± 1.3	60.7 ± 2.1

* All animals operated on at 35 days of age and sacrificed at 65 days.
† N = Nonoperated. H = Hypophysectomized.
‡ Mean ± stand. error.

permit the animal to breathe intermittently; however, it has been our experience that the attendant manipulation precipitates rupture of the delicate blood vessels.

All mice of the different strains after hypophysectomy show similar retardation of general body growth, inhibition of skeletal growth, and a marked degree of atrophy of the adrenals, ovaries and uteri. However, strain differences do become apparent during the postoperative period. Mice of the C3H and A/He strains require resuscitation in an oxygen-CO_2 atmosphere if better than 50% survival is to be expected. In addition, the latter strain must be supplied with an adrenal steroid following the operation and at regular intervals thereafter. 50% of the hypophysectomized females of the A/He strain not injected with hydrocortisone died within the first 24 hours and 80% were dead within 48 hours, whereas none of the hypophysectomized mice treated with hydrocortisone succumbed; 0.25 mg of hydrocortisone given subcutaneously as a saline suspension every tenth day was found to be sufficient to maintain this sensitive strain. C57BL, C3H, BALB/c and Swiss albino mice seem to survive indefinitely without further hormonal injections.

Summary. A practical procedure for mouse hypophysectomy by the parapharyngeal approach has been developed. Innovations include tracheal cannulation, resuscitation in an O_2 atmosphere, and hydrocortisone therapy. Mice of the A/He strain are particularly vulnerable to the effects of pituitary loss and will survive only if they are supplied with an adrenal steroid at regular intervals. On the other hand, mice of the C57BL, C3H, BALB c and Swiss albino strains do not require special postoperative handling.

1. Selye, H., Collip, J. B., and Thomson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1933, v31, 82.

2. Leblond, C. P., and Nelson, W. O., *Bull. d'histol. appl. à la Physiol. et à la Pathol.*, 1937, v14, 181.

3. Thomas, F., *Endocrinology,* 1938, v23, 99.

4. Smith, P. E., *Am. J. Anat.*, 1930, v45, 205.

5. Selye, H., *Textbook of Endocrinology,* 2nd ed., 1949, Acta Endocrinol., Inc., Montreal, p. 232.

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Fatty Liver Induced by Orotic Acid Feeding. (22005)

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In the course of a study of the effects of orotic acid, a normal component of milk, on the nutrition of young rats, it was observed that several animals developed fatty livers(1). The present study was undertaken to determine whether this effect can be consistently elicited. Since orotic acid is known to be the normal precursor of thymine and the methyl group of thymine is derived from the "C_1 pool"(2), it seemed possible that fatty livers induced by orotic acid derive from depletion of the available supply of methyl groups, analogous to the effects of nicotinamide(3) and glycocyamine(4). This possibility was tested by including various lipotropic agents in orotic acid containing diets.

Experimental. Male rats of a locally maintained strain(5), weighing 40-45 g, were placed on the various experimental diets, in groups of 10 rats each. The basal diet was as follows: casein 18%, salts(6) 5%, sucrose 75%, corn oil 2%. To each 2 kg batch of diet was added the following vitamin supplement: cod liver oil 10 g, thiamine hydrochloride 25 mg, inositol 500 mg, pyridoxine hydrochloride 25 mg, riboflavin 50 mg, calcium pantothenate 125 mg, niacin 500 mg, *p*-aminobenzoic acid 250 mg, methylnaphthoquinone 25 mg, biotin 2 mg. In addition, all diets contained 0.2% of sulfadiazine to limit the activity of the intestinal flora. The diets of various groups, as shown in Table I, contained also the following special supplements: folic acid 5 mg/2 kilo, vit. B_{12} 1 mg/2 kilo, methionine 0.8%, choline 0.3%, thymine 1%, uracil 1%. The animals were sacrificed after 28 days and liver fat was determined by a method described previously (7). The results are summarized in Table I.

Discussion. Although the mechanism remains obscure, it is apparent in Table I that the presence of orotic acid in the diet of the rat results in the accumulation of liver fat. Analysis of the lipid mixture from such livers revealed that the increase is almost entirely in the neutral fat component, much as in the fatty livers of choline-deficient animals. The fatty livers do not appear to relate to some general action of pyrimidines *per se*, since neither uracil nor thymine induced fat accumulation. The failure of folic acid, cobalamine, methionine and choline to prevent the liver fat accumulation induced by orotic acid feeding would appear to eliminate the possibility that orotic acid, by forcing thymine synthesis, creates so serious a drain on the "C_1" or methyl group supply that choline deficiency results. Indeed, the most heavily fatty livers observed in the entire series were

TABLE I. Effect of Orotic Acid and Lipotropic Factors on Liver Fat.

Orotic acid, %	Special supplement	Wt gain, g/day	Liver wt, g	Liver fat, % (wet basis) Mean ± S.E.	Liver fat, % (wet basis) Range
0	0	2.9	7.07	5.8 ± .8	4.3– 7.2
.2	0	2.8	6.02	9.2 ± 1.7	5.2–13.8
.5	0	2.5	7.94	10.9 ± 1.3	7.5–15.3
1.0	0	2.4	9.07	13.6 ± 1.4	8.3–17.0
0	Folic, B_{12}	3.0	6.50	5.9 ± .8	3.9– 9.7
.2	*Idem*	3.1	7.57	4.9 ± .6	3.6– 6.3
.5	"	3.1	9.70	9.7 ± 1.9	5.5–17.3
1.0	"	2.8	9.50	10.1 ± 2.3	5.8–14.8
1.0	Methionine	1.9	5.36	12.7 ± 1.6	7.6–16.1
1.0	Choline	2.4	8.82	16.8 ± 1.7	11.1–22.9
0	Thymine	2.6	6.73	5.5 ± .5	4.2– 6.2
1.0	"	1.8	7.24	13.4 ± 1.4	7.8–21.6
0	Uracil	2.8	7.23	4.8 ± .7	3.3– 6.5

those exhibited by rats receiving both orotic acid and choline. No alternate explanation of the effect of orotic acid on the neutral fat content of the rat liver is apparent at this time.

Summary. 1. Inclusion of orotic acid in the diet of young rats results in fatty liver formation. 2. Uracil and thymine, known to be synthesized from orotic acid, are without effect on liver fat content. 3. This effect of orotic acid is not counteracted by lipotropic factors such as folic acid, cobalamine, methionine or choline.

1. Standerfer, S. B., unpublished data.
2. Buchanan, J. M., and Wilson, D. W., *Fed. Proc.*, 1953, v12, 646.
3. Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, v146, 337.
4. Stetten, D., Jr., and Grail, G. F., *ibid.*, 1942, v144, 175.
5. Wolfe, J. M., Bryan, W. R., and Wright, A. W., *Am. J. Cancer*, 1938, v34, 352.
6. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1936, v31, 141.
7. Handler, P., *J. Biol. Chem.*, 1948, v173, 295.

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Production of Type Specific Antisera to Poliomyelitis Viruses in Rabbits.* (22006)

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The objective of this study was the production for experimental purposes of anti-poliomyelitis sera of high titer, with a high degree of type-specificity. The report of Hirst and Gotlieb(1) of the production of highly specific antisera to influenza viruses by intravenous injection of rabbits suggested that this approach might well serve our purpose. Levinson *et al.*(2) have reported the production of antisera capable of neutralizing poliomyelitis viruses, by intravenous or intramuscular injection of rabbits. They refer to earlier experience of other investigators in producing such neutralizing antibody in the serum of non-susceptible animals.†

The results reported here consist of an extension of the findings of Levinson's group, by the use of smaller doses of virus as antigen given by the intravenous route. In addition, a direct effect of the antisera alone on kidney cell culture has been observed. The main objective was achieved by repeated intravenous injections of rabbits with small doses of each of two types of poliomyelitis viruses grown in tissue culture. The titer of antibody to the same type of virus as that used for antigen (homotypic) as well as to the other type of virus (heterotypic) have been determined by neutralization tests in tissue culture.

Materials and methods. Type 1-Brunhilde and Type 2-YSK poliomyelitis viruses (obtained from Dr. Joseph L. Melnick, Yale University) which had been grown in either monkey testicular or human uterine tissue culture were used as antigens to start immunization of rabbits and for a booster dose at 5 months. The 50% endpoints (negative logarithm) of the cytopathogenic action of the viruses in HeLa culture were approximately 5 and 4 respectively. For booster doses, at 6 months or more from the beginning of immunization, Type 1-Mahoney and Type 2-MEF1 viruses grown in monkey kidney culture (obtained from Connaught Laboratories, Toronto, Canada) were used. The 50% endpoints of viral action in monkey kidney culture were 6.4 and 5.3 respectively. Supernate from a lightly centrifuged culture served as

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† By personal communication, Dr. Hubert Malherbe in the laboratory of Dr. John F. Enders has also reported the production of neutralizing antisera to poliomyelitis viruses, by intramuscular injection of rabbits with virus incorporated in oily adjuvants.

TABLE I. Development of Type 1 Neutralizing Antibody in Serum of Rabbits after Repeated Intravenous Injections of Type 1 Poliomyelitis Virus.

Interval since beginning immun. (mo):	1	5¾	6¾	8¼	14⅔	15
" " last booster (wk):	—	3	2	1	—	1½
Total No. of doses:	6	7	8	9	9	10
	Reciprocal of dilution of serum at 50% endpoint of viral effect					
Rabbit No. 10	20	30	400	1800	Dead	
11	20	5	110	600	14	180
12	20	Dead				
14	30	330	380	1600	140	680
Log_{10} ED_{50} Mahoney virus in test:	2.4	2.3	1.9	2.1	2.0	2.0

antigen for injection. In neutralization tests, the Mahoney and MEF1 viruses were used. Equal parts of virus and serum dilution to be tested were mixed and incubated for one hour at 35°C. 0.1 ml of the mixture was added to 0.5 ml nutritive medium in a tube containing one-week-old monkey kidney cells cultured directly on glass(3). (Screw-capped pyrex glass tubes which had been inoculated with approximately 50000 monkey kidney cells were received from Microbiological Associates, Bethesda, Md.) Occasionally, confirmatory tests were made in HeLa cell culture(5), usually 4 days after inoculation of pyrex glass test tubes with approximately 30000 cells. Final observation of viral action was made at 5 or 7 days, depending on the condition of the control cultures, without added virus. The amount of virus introduced in all but one neutralization test was within the range of 50 to 250 ED_{50} (effective doses); *i.e.*, $10^{1.7}$ to $10^{2.4}$ times the amount of virus at the 50% endpoint of cytopathogenic action of virus in simultaneous titration in normal rabbit serum. Dilutions of virus in titration and of antiserum for determining neutralization endpoints were made at 0.5 log increments. Titrations were done in 3 to 6 replicate tubes. 50% endpoints have been calculated according to Reed and Muench(4). Serum endpoints are reported as the dilution of antiserum that was mixed with an equal volume of virus. Some values given in the tables represent the geometric mean of endpoints found in 2 or 3 tests done on different days.

Immunization schedule. New Zealand white rabbits weighing 2-3 kg were injected intravenously. Two groups of 5 rabbits each were given 6 doses of 2 ml tissue culture virus of Type 1 or Type 2 as 3 doses a week for 2 weeks. At 5 months from the beginning of immunization, a booster dose of 1 ml was given. At approximately 6, 7 and 8 months, 2 ml each, and at 15 months, 3 ml booster doses of virus grown in monkey kidney culture were given. The rabbits were bled by cardiac puncture before, and 1 month after the beginning of immunization. They were bled also from 1 to 3 weeks after each booster dose. No anaphylactic reactions were observed. The rabbits continued to gain weight during the course of immunization. Deaths which occurred were not related to time of injection.

Results of homotypic neutralization tests. In each neutralization test, a "normal" serum control was included. This consisted of several pools of serum from the same rabbits taken before immunization, mixed with the same amount of virus as in the test. When the normal serum pool was tested "undiluted" there was no evidence of neutralization. A more sensitive test for the presence of any neutralizing capacity of pre-immunization serum consisted of titration of virus mixed with undiluted serum, either compared simultaneously with virus titrated in Earle's solution or with the expected titer based on 21 titrations with Mahoney, and on 22 titrations with MEF1 virus. With Mahoney virus, 5 separate titrations in monkey kidney culture and with MEF1 virus, 4 titrations in monkey kidney (and 1 in HeLa culture) yielded the expected, or observed, equal titer. Thus there was no evidence of any neutralizing effect of the serum of these rabbits before immunization.

TABLE II. Development of Type 2 Neutralizing Antibody in Serum of Rabbits after Repeated Intravenous Injections of Type 2 Poliomyelitis Virus.

Interval since beginning immun. (mo):	—	1	5½	6½	8	14⅔	15
" " last booster (wk):	—	—	3	2	1	—	1½
Total No. of doses (2 ml each):	0	6	7	8	9	9	10
	Reciprocal of dilution of serum at 50% endpoint of viral effect						
Rabbit No. 2		30	280	1700	5600	Dead	
15	0	100	300	5000	2500	400	560
16	0	10	350	2800	2000	560	1000
17		30	Dead				
18		100	"				
Log_{10} ED_{50} MEF1 virus in test:	1.8	1.9	2.0	1.9	1.8	1.8	1.8

Table I shows the neutralizing capacity of anti-Type 1 serum vs. homotypic Mahoney virus. Antibody was demonstrable in all 4 rabbits at 1 month, the level rising with 3 booster doses up to 8¼ months after the beginning of immunization when endpoints of serum of the 3 surviving rabbits ranged from 600 to 1800. During a subsequent rest period of 6 months, endpoints fell although antibody was still demonstrable. A subsequent booster dose in the 2 remaining rabbits raised the endpoints to moderately high levels, although not quite up to the maxima observed at 8 months.

Table II gives the endpoints of neutralization of anti-Type 2 sera vs. homotypic MEF1 virus. All 5 rabbits showed an antibody response of from 10 to 100 by the end of the first month. With 2 or 3 subsequent booster doses, levels rose to give endpoints at 8 months of from 2000 to 5600. After a rest period of nearly 7 months, antibody fell to moderate levels in the 2 surviving rabbits and rose somewhat again after one more antigenic stimulus.

Results of heterotypic neutralization tests. Table III gives the results of cross neutralization tests. The sera of 3 rabbits before immunization with Type 2 poliomyelitis virus showed no effect on Type 1 virus. By 6½ months after beginning of immunization, after 2 booster doses, sera of 2 of 3 rabbits showed endpoints of 10 each, the third, 0. An additional booster by 8 months did not increase this cross reaction. (The homotypic endpoint ranged from 2000 to 5600 in these three samples of serum.) None of 3 anti-Type 1 antisera (see Table III) showed heterotypic antibody by nearly 6 months, after 6 doses of virus plus a booster dose. After a second and third booster dose, at approximately 7 and 8 months, 1 (or possibly 2) of 3 sera showed a minimal amount of cross neutralization. The homotypic endpoints of these sera at 8¼ months ranged from 600 to 1800. Later samples of sera were not tested vs. heterotypic virus because of a complicating reaction which will be described in the next section.

Immuno-cytopathogenic effect of antiserum. Since the initial series of injections as well as the first booster dose in the rabbits consisted of virus grown in human uterus or in monkey testis, the possibility of a reaction of the antisera directly on the monkey kidney cells was not as great as if the virus had been grown in the same type of cells as that used in the neutralization test. Indeed, the antisera being studied for antiviral activity showed no direct effect on monkey kidney cells until the 7 and 8 months' samples, after the rabbits had received their first exposure to virus grown in monkey kidney culture as 1 and 2 additional booster doses. The serum of one (#10) of the 6 rabbits remaining showed cytopathogenic effect at a 1/6 final dilution of serum; *i.e.*, 0.1 ml undiluted serum added to 0.5 ml culture fluid. After the third booster dose of monkey kidney grown virus at 15 months, sera of all 4 rabbits surviving produced an untoward effect on monkey kidney cells in culture. The reaction consisted of either lysis of agglutination of the cells, resulting in the extreme in their complete destruction or their falling off the wall of the test tube. The reaction is comparable to that observed when HeLa cells were exposed to antiserum prepared in rabbits by intravenous injection of HeLa cell constituents(6). The

TABLE III. Degree of Cross Type Neutralization of Anti-Poliomyelitis Sera Produced in Rabbits.

		Anti-Type 2 serum vs. Type 1 (Mahoney) virus					Anti-Type 1 serum vs. Type 2 (MEF1) virus		
Interval since beginning immun. (mo):		—	5½	6½	8		5¾	6¾	8¾
" " last booster (wk):		—	3	2	1		3	2	1
Total No. of doses:		0	7	8	9		7	8	9
	Rabbit No.					Rabbit No.			
	2	0	0	0	0	10	0	10	3
	15	0	0	10	2	11	0	2	0
	16	0	10	10	10	14	0	0	0
Log_{10} ED_{50} virus:		2.1	1.7	2.2	2.4		1.7	1.9	2.8

addition of neutral red at a final dilution of 1/100,000, observed to be compatible with healthy cells, helped to visualize the reaction. One antiserum (rabbit #14, 15 months' sample) was tested repeatedly, yielding an average 50% endpoint of 30 of anti-kidney cell reaction. The endpoint of homotypic virus neutralization by this serum was 680.

The possibility of a cytopathogenic action on cells in culture not due to virus must be taken into account when carrying out neutralization tests, especially with high concentrations of antiserum such as used in heterotypic tests. Unless the antiserum alone is shown to have no effect on the cells in culture, this anti-cellular reaction could be mistaken for lack of cross neutralization.

Summary. A simple and inexpensive method for producing antisera with a high degree of neutralizing capacity for poliomyelitis viruses has been described. By repeated intravenous injection of rabbits with small doses of poliomyelitis viruses grown in tissue culture, antisera with a high degree of homotypic with little or no heterotypic neutralizing capacity have been produced regularly. Such antisera develop also to a lesser degree the capacity to lyse or agglutinate the cells in culture, in the absence of virus. When testing for heterotypic neutralization, this reaction may mask cross neutralization.

1. Hirst, G. K., and Gotlieb, T., *J. Exp. Med.*, 1953, v98, 41.

2. Levinson, S. O., Milzer, A., Shaughnessy, H. J., Wolf, A. M., Janota, M., Vanderboom, K., Neal, J. L., and Morrissey, R. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 111.

3. Morann, G. L., and Melnick, J. L., *ibid.*, 1953, v84, 558.

4. Reed, L. J., and Muench, H., *Amer. J. Hyg.*, 1938, v27, 493.

5. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

6. Mountain, I. M., *J. Immunol.*, in press.

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Effects of Reserpine and Chlorpromazine on Nest Building and Thermal Preference in Rats. (22007)

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Reserpine and chlorpromazine have somewhat similar effects on animal behavior. Both drugs depress general activity(1,2) and reduce responsiveness to environmental stimuli(3-6). Both drugs also tend to cause hypothermia (1,4,8). This study attempted to determine whether two forms of basically unlearned motor behavior related to low temperature could be influenced by moderate amounts of these drugs. Hypothermia resulting from drug treatment might be expected to cause preference for a warm environment and increased nest building in rats; but general sedation, if marked, would tend to depress both nest building and active search for the preferred thermal environment.

Methods. Twenty-four male albino rats of Wistar stock, 120-180 days old and 275 g average weight were used. Thermal preference was measured in an apparatus consisting of 2 interconnecting plywood units (5 x 5 x 32 in.) in which the temperatures could be independently regulated by means of strip heaters(7). The temperatures were set at 14-18°C (room temperature), and 32-36°C, alternated each day with respect to unit heated. Illumination was provided by an overhead light. Each animal was given a daily test in the apparatus lasting 4 minutes. Every 10 seconds during the last 3 minutes of the period a notation was made of the unit in which the animal appeared. Preference was measured in terms of the frequency with which the animal appeared in the warmer unit. Thus a score from 0-18 was possible. Animals were maintained in large nesting cages (24 x 18 x 12 in.) with food and water always available. Nesting material was provided in the form of 150 g of wood shavings and 50 g of paper stripping. Each evening previous nests were broken up and the nesting material scattered throughout the cage. On the following morning the nests were rated independently by the experimenter and a second person, using the scale of Stone and Walker(9). Ratings range from 0 for no evidence of a nest to 5.0 for an excellent nest. Room temperature was reduced to 10-14°C during the night to promote nesting and raised to 25°C between nesting periods. The first part of the experiment was a pre-drug phase of 4 days. Groups of animals equated on a basis of nesting performance were then assigned to the experimental treatments indicated in Table I. During this phase, subcutaneous injections of the drugs were given one hour before start of the nesting period. Reserpine was given as .25% solution in a propylene glycol base, chlorpromazine as 2% solution in isotonic saline.

Results. The results for nesting and thermal preference are shown in Fig. 1. The data were treated by 2-way analyses of variance in which effects of each drug and their possible interactions could be segregated. Pre-drug data showed no statistically significant group differences but thermal preference behavior was selective in that all animals preferred the cooler unit.

As shown by Fig. 1, reserpine affected both thermal preference and nesting behavior much more strongly than chlorpromazine. On the 3rd and 4th days of reserpine treatment (groups R and ChR) nest building was effectively abolished ($p < .001$) and a reversed preference for the warmer environment was established ($.05 > p > .02$; significance of mean frequency above expected chance frequency of 9). Chlorpromazine (groups Ch and ChR) inhibited nesting following the first and second injections. After the second day the apparent slight depression of nesting was of doubtful statistical significance ($p = .05$). Chlorpromazine alone had no effect on thermal preference. Disturbed thermal preference was evident for several days after discontinuing re-

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TABLE I. Experimental Groups.

Group	N	Treatment	Dose
R	6	Reserpine	.5 mg/kg for 3 days; .25 mg/kg on 4th day
ChR	6*	Chlorpromazine	10 mg/kg for 4 days
		Reserpine	.5 mg/kg for 3 days; .25 mg/kg on 4th day
Ch	6	Chlorpromazine	10 mg/kg for 6 days; 15 mg/kg for 2 days
C	6	Isotonic saline	Comparable volumes to group Ch

* All data discarded for one animal that died after first inj.; data for a second animal that died on 5th day was included in the analyses.

serpine but nest building recovered within one day.

Weight changes during nesting periods were also measured. Declines in weight, presumably reflecting reduced food intake, accompanied the depressed nesting. Prompt recovery of weight followed discontinuance of reserpine injections.

Discussion. Both reserpine and chlorpromazine affected nesting behavior and weight loss but, with the doses used, the effects of reserpine were clearly much greater. Reserpine

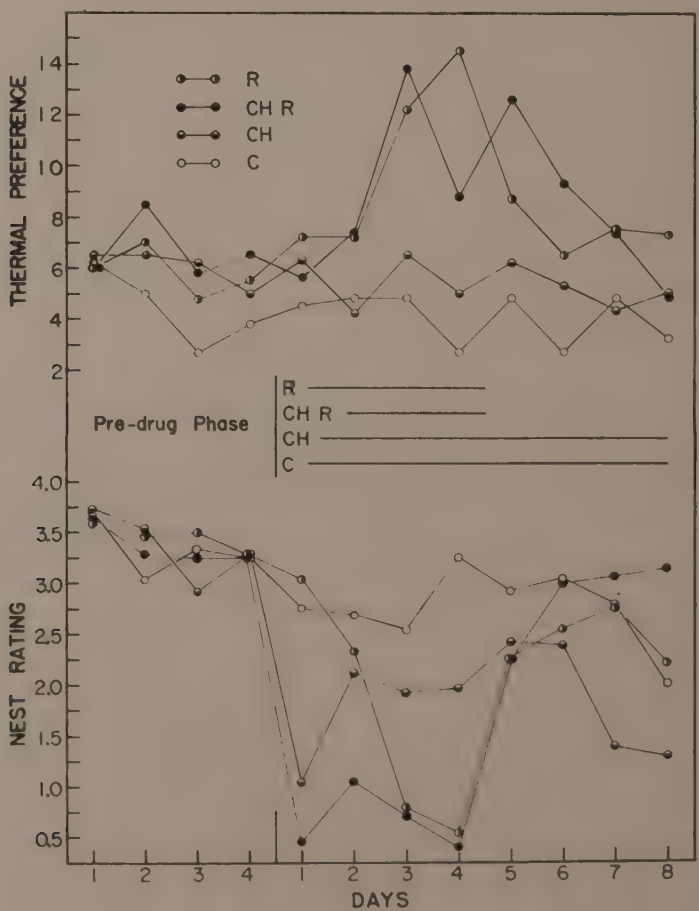

FIG. 1. Mean thermal preference scores and nest ratings.

alone affected thermal preference. Slight indications of synergic effects of the 2 drugs were seen but not statistically verified. The persistence of preference for a warmer environment for several days after discontinuing reserpine injections might be due to a persistent drug effect or to a persistence of a behavior learned during the drug phase.

It is of interest that reserpine greatly depressed one adaptive behavioral reaction (nest building) to the hypothermic state presumably resulting from the doses used but promoted another (choice of a warm environment). This difference may be related to the marked differences in the nature of the behavioral responses tested and the methods of testing. The thermal preference test involved arousal of the animal by handling, which could be expected to last throughout the 4-minute test period; nest building required long persistent activity under conditions of minimal disturbance. Reserpine-treated animals were easily aroused and quite active while awake but readily fell into a deep sleep when not disturbed. The experiment does not exclude, however, the possibility of a differential drug effect on mediational mechanisms of the two behaviors. The implications for behavioral research with reserpine in terms of distinguishing between sedative effects of the drug and other more specific actions are apparent.

Summary. Effects of reserpine, chlorpromazine and combined drugs on nest building and thermal preference of rats are described. In the doses used, reserpine alone had a marked and persistent effect on these functions. It depressed nest building while promoting choice of a warm environment. The differential behavioral effect is thought to be related to the great differences in persistence of activity required for the two tests and the ability of reserpine-treated animals to maintain alertness for brief periods after handling.

1. Tripod, J., Bein, H. J., and Meier, R., *Arch. Intern. Pharmacodynamie,* 1954, v96, 406.

2. Boyd, E. M., and Miller, J. K., *Fed. Proc.,* 1954, v13, 338.

3. Chusid, J. G., Kopeloff, L. M., and Kopeloff, N., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 276.

4. Plummer, A. J., Earl, A. E., Schneider, J. A., and Barrett, W., *Ann. N. Y. Acad. Sci.,* 1954, v59, 8.

5. Schneider, J. A., *Bull. Med. Research,* 1954, v9, 10.

6. Schneider, J. A., and Earl, A. E., *Neurology,* 1954, v4, 657.

7. Mason, W. A., The effects of hypophysectomy and electroconvulsive shock on thermal preference and nesting in rats, Ph.D. Thesis, Stanford U., 1954.

8. Ripstein, C. B., Friedgood, C. E., and Solomon, N., *Surgery,* 1954, v35, 98.

9. Stone, C. P., and Walker, A. H., *J. Comp. Physiol. Psychol.,* 1949, v42, 429.

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Total Plasma Ascorbic Acid as Index of Stress in the Rat. (22008)

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One of the manifestations of physiologic stress is an increase in the production by the adenohypophysis of adrenocorticotrophin (ACTH) (1). A temporary rise in the concentration of total plasma ascorbic acid and plasma ascorbic acid following administration of ACTH in man has been reported (2).

As a preliminary to further experiments, it was deemed desirable to compare the sensitivity of this index of stress and possible ACTH titer in the blood of albino rats with other such indices.

Methods. Animals in one group were anesthetized with sodium-ethyl-methyl-butyl-barbiturate (Nembutal) for 2 hours. Those in Group 2 were anesthetized with Nembutal for 2 hours and during this time non-activated electrodes were kept in contact with the left cervical sympathetic trunk just posterior to the superior cervical ganglion. The third group was treated like Group 2 except that the electrodes were activated so that the sym-

TABLE I. Average % Increase or Decrease following Treatment Indicated.

Component of blood	Nembutal only	Nembutal with non-activated electrodes	Nembutal with stimulation
Ascorbic acid	+14.15 (13)*	+17.97 (18)	+23.86 (15)
Eosinophils	−10.39 (20)	−25.50 (20)	−42.89 (31)
Lymphocytes	−31.27 (17)	−37.55 (17)	−43.84 (29)
Neutrophils	+55.26 (18)	+66.67 (17)	+56.22 (30)

* Figures in parentheses = No. of animals.

pathetic trunk was receiving .05 to .07 volt impulses of 20 milli-second duration from a square wave stimulator 20 times every second. Prior to attachment of the electrodes, a section of the vagus nerve in the region being stimulated was removed from animals in Group 2 and 3 to avoid death from cardiac or respiratory failure during the 2-hour period of treatment. Stimulated animals that did not exhibit sustained expansion of the pupil on the activated side were discarded. Blood counts and total plasma ascorbic acid determinations were made before and after treatment. Blood was usually taken from the tail. It was taken from the heart in only 4 cases. These 4 were giving excellent results but enough blood could not be obtained from the tail. The leucocyte count of blood taken from the heart differs from that of blood taken from the tail(3). The Roe and Kuether method of determining plasma ascorbic acid was used (4). Diet and temperature were maintained relatively constant. The experiments were started at the same time every day. Rats used were young adult males, all about the same age and weight, from the E. A. Doisy Biochemical Laboratories, Saint Louis University Medical School.

TABLE II. Percent of Animals Treated Showing an Increase or Decrease in Blood Components.

Component of blood	Nembutal only		Nembutal with non-activated electrodes		Nembutal with stimulation	
	Incr.	Decr.	Incr.	Decr.	Incr.	Decr.
Ascorbic acid	100		100		100	
Eosinophils	40	60	10	90	10	90
Lymphocytes	12	88	12	88	7	93
Neutrophils	100		100		93	7

Results. The 3 types of treatment outlined above produce temporary manifestations of stress, and thereby possibly of ACTH titer in the blood, demonstrable by each of the criteria used. Such manifestations are eosinopenia, lymphopenia, neutrocytosis and an increase in the total plasma ascorbic acid. The average percent changes in the eosinophil and lymphocyte counts and in the plasma ascorbic acid determinations, indicate that animals with non-activated electrodes attached were subjected to greater stress than were those receiving Nembutal only, and that activation of the electrodes caused the most severe stress (Table I). The average percent increase in neutrophils was not as great in the stimulated animals as it was in animals having non-activated electrodes attached (Table I). Therefore, the neutrophil count is probably not a reliable index of ACTH titer in the blood. This conclusion is upheld by other workers (5).

TABLE III. Levels of Significance (P) of Differences between Methods of Treatment Indicated.

Component of blood	Nembutal only and Nembutal with non-activated electrodes	Nembutal with non-activated electrodes and Nembutal with stimulation	Nembutal only and Nembutal with stimulation
Ascorbic acid	.343	.105	.046
Eosinophils	.018	.015	<.001
Lymphocytes	.370	.393	.083
Neutrophils	.087	—	.809

From Table II it is seen that during 2 hours of treatment, all the animals in each of the 3 groups show an increase in plasma ascorbic acid, whereas a few from each group paradoxically show eosinophilia and lymphocytosis.

Thus it would appear that plasma ascorbic acid is the most reliable index of stress even though statistical analysis of the overall results (Table III) points to eosinopenia as the most sensitive indicator.

Summary. The effect of electrical stimulation of the cervical sympathetic trunk on the number of eosinophils, lymphocytes and neu-

trophils and on the total plasma ascorbic acid in the blood of the rat, indicates that the amount of total plasma ascorbic acid rises temporarily in these animals following this type of stress and that it is a reliable index of the severity of such stress and possibly of ACTH production following such stimulation.

1. Selye, H., *Canad. M. A. J.*, 1944, v50, 426.
2. Stewart, C. P., Horn, D. B., and Robson, J. S., *Biochem. J.*, 1953, v53, 254.
3. Goldie, H., Jones, A. M., Ryan, H., and Simpson, M., *Science*, 1954, v119, 353.
4. Consolazio, C. F., Johnson, R. E., and Marek, E., *Metabolic Methods*, 1st ed., 1951, C. V. Mosby, Saint Louis, pp. 198-199.
5. Dougherty, T. F., and White, A., *Endocrinology*, 1944, v35, 1.

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Effect of Intrinsic Factor Concentrate on Vit. B_{12} Absorption by Gastrectomized Rats. (22009)

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A recent attempt(1) to use normal rats to test the potency of an intrinsic factor preparation revealed only an inhibitory effect on the absorption of oral vit. B_{12} by this test animal. Normal rats had been employed for this study which involved doses of 4.2 and 0.42 μg of radioactive vitamin labeled with cobalt 60.

Normal rats do not develop a macrocytic anemia comparable to pernicious anemia, and may thus be considered *a priori* unsuitable for the evaluation of intrinsic factor potency. In humans, however, gastrectomized individuals (2,3) have been employed successfully to demonstrate intrinsic factor despite the absence of anemia or megaloblastic marrow. This suggested the possibility that gastrectomized rats would serve as appropriate test animals. Accordingly studies were undertaken to examine the effect of potent intrinsic factor concentrates upon the extent of absorption of radioactive vit. B_{12} by gastrectomized rats. Furthermore, physiological doses of 200 mμg and 50 mμg of vitamin, and 2 different intrinsic factor concentrates, were investigated.

Methods. Adult rats of different strains weighing 200-300 g were used. Upon open ether anesthesia total gastrectomy was performed utilizing an end to end esophagoduodenostomy to restore intestinal continuity. Fine silk was used for sutures and ligatures throughout. Total removal of the stomach was verified in each instance by histological preparation of a saggital section through the removed organ. In general at least 10 days were allowed for recovery from the operation before transfer of the animals to the nutrition laboratory. During the first 10 days after operation, the animals were fed on a milk and bread diet and lost about 10% of pre-operative body weight. Subsequently, they were offered a basal soybean diet, and during test periods were given 10 g of this diet daily.

Materials. Two commercially available intrinsic factor concentrates were employed. Preparation I was used only in preliminary study (Series A) with low specific activity vit. B_{12}-Co^{60}. Its clinical potency was not retested. Preparation II was of proven potency at 40 mg, as tested hematologically and by the Schilling method(4), and at 20 mg by the latter procedure. Its binding power was determined by the tracer-dialysis method(5) to be 250 mμg/mg. The vit. B_{12} labeled with Co^{60} employed in the preliminary study had a specific activity of $\simeq$ 55 μc/mg; and the activity of the second preparation, used in 3 additional definitive studies, was $\simeq$ 200 μc mg.

Method. Four series of experiments (A, B, C and D) were performed in such manner

TABLE I. Summary of Fecal Excretion Results—Gastrectomized Rats vs Controls.

Series	mμg B_{12}	mg IF	Phase	Duration (days)	No. of animals Gastrectomized	No. of animals Control	Group avg % of dose excreted Gastrectomized	Group avg % of dose excreted Controls
A	200	0	I	5	1	2	107	71 ± 6
		10	II	4	1	2	109	95 ± 3
B	200	0	I	7	4	4	102.0 ± 5.8	84.9 ± 5.1
		3	II	2	4	4	96.4 ± 2.6	>82.9 ± 6.4
C	50	0	I	8	4	4	101.7 ± 4.1	62.4 ± 14.5
		15	II	4	4	4	100.5 ± 2.9	97.5 ± 2.8
D	50	0	I	6	6	6	99.4 ± 5.0	50.0 ± 13.6
		.2	II & III	6	5*	6	104.6 ± 3.0	71.0 ± 17.2
		1	III & II	6	4*	6	99.9 ± 4.7	98.6 ± 3.5

* One animal was found dead before conclusion of this phase.

that each rat served as its own control. After a 24-hour basal collection period, each animal received a 50-200 mμg of Co^{60} vit. B_{12} by stomach syringe, and feces collected daily until fecal radioactivity was negligible (Phase I). Then the same dose of tracer was administered simultaneously with a fixed amount of intrinsic factor concentrate, and collections continued as before (Phase II). In one series (D) a third phase involving administration of additional intrinsic factor was introduced. In this series, control and gastrectomized rats were each divided into 2 groups for Phase II, one group receiving 0.2 mg of concentrate, the second group receiving 1 mg. During Phase III, these dosages were reversed. In Series A, animals were fed milk to flush out their bowels at the conclusion of Phase I. Radioactivity measurements by gamma ray scintillation counting(1,5) were performed on feces homogenates in water suspension. Although stools were collected daily, several daily collections were combined for measurement in certain instances. Excretion was essentially complete in 3-4 days. No radiometric examination was made of rat organs.

Results of excretion measurements were calculated as "% of Dose." A large value signifies negligible absorption, while a low excretion indicates absorption. Basal periods are not reported since activities were zero. Basal corrections for Phases II and III were based upon the average activity (usually negligible) excreted for the 2 days immediately preceding these phases.

Group average excretion values (± average deviation from the mean) are compiled in Table I for each of the 4 series. Also shown are the doses of vit. B_{12} and intrinsic factor administered, the duration of each phase and the number of animals employed. Series D excretions listed for phases II and III actually represent averages for 0.2 and 1.0 mg intrinsic factor fed in both phases.

Discussion. In none of the 4 series did the gastrectomized rats absorb significant amounts of vit. B_{12} within our experimental error, regardless of the presence of intrinsic factor; and in Series A, C and D, intrinsic factor reduced absorption by the control rats to essentially zero. This inhibitory effect of intrinsic factor is not shown in Series B control animals, in part because fecal collections during the second phase (plus intrinsic factor) was discontinued after the second day. Undoubtedly further excretion of radioactivity would have been noted had the experiment been continued. Furthermore, it is questionable whether complete inhibition could be attained in this experiment in view of the low ratio (3.75) of intrinsic factor binding capacity to B_{12} dose.

Series C and D were performed with 50 mμg of radioactive vitamin since the percentage absorption would be greater than at the 200 mμg level, and because 50 mμg represents a physiological dose equivalent weight-wise to 10-12 μg per adult human. In these experiments it was established unequivocally that gastrectomized rats absorb only a negligible fraction of the oral vitamin, whether or not intrinsic factor is administered, and that intrinsic factor inhibits absorption of the vitamin by the controls.

In Series C, the quantity of intrinsic factor concentrate employed (15 mg) was disproportionately large, but was reduced in Series D to 1.0 mg and 0.2 mg, the latter amount being calculated just to bind completely the oral vit. B_{12} dose (*i.e.* binding power 250 mμg/mg or 50 mμg/0.2 mg). Complete inhibition of absorption occurs even with 1 mg of concentrate; and at 0.2 mg inhibition is still evident though to a reduced extent. It appears that complete inhibition of vit. B_{12} absorption occurs when the ratio (IF binding capacity) (vit. B_{12} dose) is approximately 5. Until such an excess capacity is established, inhibition increases gradually with increasing quantity of intrinsic factor.

Obviously, the rat, gastrectomized or normal, is not a suitable animal for testing intrinsic factor activity, which causes only a diminished absorption in normal rats and which has no effect on gastrectomized animals. It is possible that the rat can utilize only endogenous intrinsic factor and that intrinsic factor of foreign origin is ineffective and inhibitory. The behavior of the gastrectomized animals may also indicate that the stomach is the major seat of vit. B_{12} absorption by the rat. These points are to be tested by experiments with rat stomach concentrate. If this concentrate proves to be effective, it will demonstrate species specificity in the action of intrinsic factor.

The livers of gastrectomized rats contain an abnormally low vit. B_{12} content, as would be expected from their inability to absorb appreciable quantities of the vitamin orally.

On a weight basis, the rat appears capable of absorbing greater quantities than the human. Thus, in the absence of intrinsic factor, absorption from an oral dose of 50 mμg amounts to 20-25 mμg; and from 200 mμg, absorption is 30 mμg. The equivalent dose figures for a 60 kg human would be 10 μg and 40 μg, and the corresponding absorption would amount to 4-6 μg. Actually the human absorbs(6) only 1.5 μg from such oral levels.

Summary. 1. Gastrectomized rats and appropriate control animals were fed 50-200 mμg cobalt-60 labeled vit. B_{12} with and without intrinsic factor concentrates. Absorption was determined by scintillation counting of feces homogenates. 2. No absorption of vit. B_{12} by the gastrectomized animals was noted. 3. Absorption by control animals was inhibited by intrinsic factor concentrate, the extent of inhibition increasing with the quantity of concentrate administered.

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1. Rosenblum, C., Woodbury, D. T., Gilfillan, E. W., and Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 268.

2. Swenseid, M. E., Halsted, J. A., and Libby, R. L., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 226.

3. Halsted, J. A., Gasster, M., and Drenick, E. J., *New England J. Med.*, 1954, v251, 161.

4. Schilling, R. F., *J. Lab. Clin. Med.*, 1953, v42, 860.

5. Rosenblum, C., Woodbury, D. T., and Reisner, E. H., Jr., *Proc. Second Radioisotope Conference*, Oxford, July 19-23, 1954, Butterworth Scientific Publications, London, 1954, v1, p287.

6. Glass, G. J. B., Boyd, L. J., and Stephanson, L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 522.

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Effect of Chlorpromazine upon Epileptic and Normal Monkeys.* (22010)

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The widespread use of chlorpromazine in treatment of disorders affecting the nervous system of man suggested the advisability of a study of its effects upon epileptic and normal monkeys.

Method. Chronic experimental epilepsy was produced in 13 *Macaca mulatta* (3-4 kg) by the application of alumina cream to a cerebral cortical area(1). Discs containing alumina cream were applied to a principal sensorimotor area in 10 monkeys; multiple injections of alumina cream were made into an occipital cortical area in 2 monkeys and into an anterior mesial temporal area in the remaining monkey. Cerebral arteries (anterior, middle cerebral or carotid) were ligated in some monkeys in an effort to modify the clinical epilepsy produced. Chlorpromazine in doses of 2 mg/kg or 10 mg/kg was injected intravenously within one minute and the animals observed subsequently for a period of 4 hours during which behavioral, clinical and electroencephalographic studies were made. A total of 40 test injections (spaced at approximately biweekly intervals) were given to the experimental group. Clinical epileptic seizures were activated by vigorous prodding for one minute or by the intramuscular injection of small doses (16 mg/kg or less) of pentamethylenetetrazole (Metrazol®). Five normal unoperated monkeys were treated with chlorpromazine on 14 occasions and served as controls. In this group the effect of vigorous prodding as well as of intramuscular Metrazol (32 mg/kg) was similarly tested(2).

Results. Profound changes in behavior following intravenous injection of chlorpromazine occurred in both epileptic and normal monkeys. Within 1-2 minutes the animals became quieter, less aggressive, lethargic, flaccid and hypokinetic. They were soon unable to maintain a sitting or standing posture, becoming progressively more stuporous; eyelids remained drooped or closed, and increased salivation and tachypnea were frequently noted. On being touched, the monkeys would occasionally make feeble efforts to blink eyelids, open mouth or withdraw a stimulated limb. Jerking myoclonic type movements of the body, tail or extremities sometimes occurred. Epileptic monkeys occasionally exhibited a typical focal or generalized motor convulsion spontaneously during chlorpromazine treatment. Maximum behavioral changes lasted 30 to 60 minutes, subsiding gradually thereafter within the next 4 to 6 hours. Effects were generally more marked and sustained with the larger doses used.

Pronounced electroencephalographic changes occurred within 1-2 minutes after the intravenous administration of chlorpromazine. Diffusely distributed slow waves with frequency of 2 to 5 per second and maximum amplitude of 150 microvolts became dominant. Against this slow wave background, there was usually noted in epileptic monkeys a striking pattern of increased spike and sharp wave discharges (Fig. 1).

Among the epileptic monkeys treated with chlorpromazine, the intramuscular injection of 16 mg/kg or less of Metrazol was uniformly effective in provoking clinical epileptic seizures. In some animals of this group, while under the influence of chlorpromazine, a smaller dose of intramuscular Metrazol was sometimes effective in provoking a seizure than was necessary in the untreated state. Normal control monkeys exhibited no clinical epileptic seizures while under the acute influence of chlorpromazine despite the use of relatively large doses of intramuscular Metrazol (32 mg/kg).

Vigorous prodding of epileptic monkeys following chlorpromazine treatment usually resulted in the activation of clinical epileptic seizures. Normal monkeys similarly stimulated while under the effect of chlorpromazine

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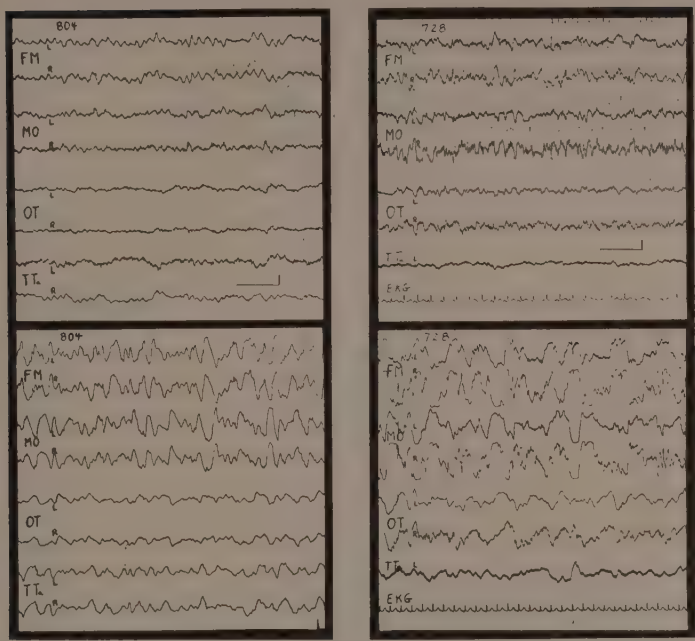

FIG. 1. Electroencephalogram before (top) and ½ hr after (below) intravenous chlorpromazine treatment (2 mg/kg). Normal monkey (804) on left; epileptic monkey (728) on right. F = frontal; M = motor; O = occipital; T = temporal; Ta = anterior temporal; EKG = electrocardiogram. Calibration: 1 sec (horizontal); 50 microvolts (vertical).

never showed evidence of clinical seizures.

Discussion. In general the behavior of the epileptic and normal monkeys following the intravenous administration of large doses of chlorpromazine did not differ significantly, and resembled that described by Das *et al.*(3) for normal monkeys. Striking EEG changes noted in chlorpromazine-treated monkeys consisted of the prompt appearance of diffuse high amplitude slow frequencies. In the epileptic monkeys, there was, in addition, increased spike and sharp wave formations. This latter effect may be similar in type to that often encountered in man, where somnolence, or natural or drug induced light sleep increases the appearance of spike discharges(4). Chlorpromazine did not protect any epileptic monkey against the seizure precipitating effect of small doses of Metrazol. Some of these animals appeared to be even more susceptible since they required a smaller threshold dose to provoke clinical seizures. Normal monkeys, however, always remained refractory to intramuscular Metrazol even with the relatively high dose of 32 mg/kg (convulsant dose for normal untreated monkeys: 48 mg/kg or greater). Significant alteration in the clinical response of epileptic monkeys to activation of seizures by vigorous prodding was not observed in these experiments.

Conclusions. 1. The rapid intravenous administration of chlorpromazine (2-10 mg/kg) to epileptic and normal monkeys produced prompt profound behavioral changes characterized by hypokinesia, flaccidity, diminished responsiveness, reduced aggressiveness, lethargy and somnolence. 2. High amplitude slow wave activity (2-5/second) became dominant in the EEG up to several hours after intravenous injection of chlorpromazine; epileptic monkeys showed, in addition, increased high amplitude spike and sharp wave forms. 3. Epileptic monkeys under the acute influence of chlorpromazine retained their clinical convulsive responsiveness to intramuscular Metrazol (16 mg/kg or less). Clinical epileptic

response to vigorous prodding was not significantly changed. 4. Normal control monkeys under the acute influence of chlorpromazine did not exhibit clinical convulsions and remained refractory to treatment with intramuscular Metrazol (32 mg/kg) and to vigorous prodding stimulation.

1. Kopeloff, L. M., Chusid, J. G., and Kopeloff, N., *Neurology*, 1954, v4, 218.

2. Chusid, J. G., Kopeloff, L. M., and Kopeloff, N., *J. Appl. Physiol.*, 1953, v6, 139.

3. Das, N. N., Dasgupta, S. R., and Werner, G, *Bull. Calcutta School Tropical Med.*, 1954, v1, 5.

4. Gibbs, F. A., and Gibbs, E. L., *Atlas of Electroencephalography*, Addison-Wesley Press, Cambridge, Mass., 1952, v2, 19.

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In vitro and *in vivo* Neutralization of the Virus of Visceral Lymphomatosis. (22011)

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It has been reported(1) that the progeny of hens which had received a series of injections of the virus of visceral lymphomatosis were much more resistant to challenge with this virus than were the progeny produced by the same hens before the injections were made. The suggestion was made that the basis for this increased resistance was the transfer of antibodies from the dam to the egg and thence to the chick. This is known to occur in other diseases of chickens.

Tests reported here provide evidence that the tumor inciting properties of the virus of visceral lymphomatosis are neutralized by specific antiserum, that serum neutralizing antibodies in chickens are produced by the injection of materials containing the live virus, and that the increased resistance of the progeny is directly related to the increase in specific serum antibodies in dams.

Materials and methods. Antisera for *in vitro* and *in vivo* neutralization tests were obtained from hens that had received a series of injections of preparations containing the virus of visceral lymphomatosis. A description of these preparations and the sequence of injections have been given(1). Normal serum for both tests was collected 6 days before the hyperimmunizing injections were begun. Immune serum for the *in vitro* test was collected from one of the hens 4 days after the last of the first series of injections. Two months later a second series of injections was given intramuscularly. The material injected was live virus preparation similar to that used in the first series. Immune serum for the *in vivo* test was collected 6 weeks after the last of the second series of injections. There was an interval of about 6 months between the first injection and the final collection of immune serum. To provide a sufficient volume of serum for the *in vivo* test the serums from 9 hens were pooled. All serums were obtained from blood collected by heart puncture. The blood was transferred to large centrifuge tubes and refrigerated over night. After centrifugation the serum was transferred to glass vials and sealed. The tubes were stored at −30°C. The source of virus used in both tests was lymphomatous livers of birds in the sixteenth serial passage of strain RPL 12(2). The chicks were from an inbred line of S.C. White Leghorns maintained in isolation for the past 13 years. The incidence of lymphomatosis in isolation has been very low; however, when naturally or artificially exposed, the stock has manifested susceptibility to the disease. *In vitro* neutralization tests were made with constant volumes and dilutions of serums mixed with 4 serial 100-fold dilutions of the virus preparation. Dilutions were made with Simms' salt solution containing 1.5% bovine albumin. Each inoculating dose of 0.2 ml contained filtrate obtained from log −3, −5, −7, and −9 g of lymphomatous liver. All serums were heated to 56°C for 30 minutes.

TABLE I. *In Vitro* Neutralization of Virus of Visceral Lymphomatosis by Specific Antiserum.

Serum	Log dose of virus	No. inoculated Total	Corrected	% visceral lymphomatosis 112	140	168	201 days	Latent period (days)
Normal	−3	42	40.7	61.5	66.4	69.8	71.3	81.0
	−5	40	35	17.1	31.4	37.2	60.0	138.2
	−7	37	36.1	8.3	24.9	33.3	36.0	129.5
	−9	41	39.4	0	0	2.5	12.7	191.8
Immune	−3	41	35.2	11.3	22.7	56.9	76.8	148.0
	−5	42	40.1	2.5	2.5	5.0	25.0	172.5
	−7	41	39	0	2.6	5.1	5.1	149.0
	−9	40	39	0	0	0	0	—
"	0	38	38	0	0	0	0	—
Diluent	0	38	38	0	0	0	0	—

24 hours before use. One ml of serum was added to each 9 ml of virus preparation. The virus preparation and the virus-serum mixtures were maintained at melting ice temperatures at all times. The interval between mixing and inoculation of the various mixtures was 2-3 hours. The inoculations were made with a 1 ml tuberculin syringe into day-old chicks by the intraperitoneal route. For *in vivo* neutralization tests, chicks were injected with pooled serum—on the first day with 0.5 ml intraperitoneally, on the second day with 1 ml subcutaneously, and on the third day with 0.5 ml subcutaneously. On the third day the chicks received by the intraperitoneal route 0.2 ml of the virus preparation of a dilution equivalent to log −3, −5, or −7 g of tumor. All chickens were maintained for a period of 201 days under experimental conditions similar to those previously described(3,4).

Results. Data obtained from the *in vitro* neutralization test are summarized in Table I. The percentage of mortality from visceral lymphomatosis was based on the number per lot as corrected(3) for deaths due to other causes. The incidence of tumors at progressive ages is given in the table, since it has been shown(4) that differences in response to different dosages tends to decrease with an increase in the holding period. With one exception, the percentage of visceral lymphomatosis for each age interval and for different virus dosage lots was lower in chickens that had received the inoculum containing immune serum than in those that had received mixtures containing the normal serum. These quantal response data clearly indicate that at the dose of log −9 practically all, if not all, of the virus was neutralized and at the doses of log −7 and −5 at least 99% of the virus was neutralized. There was no essential difference in the incidence of tumors between the immune and normal serum at the log −3 dosage level and longest holding period, but at this highest virus dosage, partial neutralization is indicated by a marked delay in mortality. If the latent period is used as the basis for virus activity, it can be estimated that the addition of immune serum to the virus at a log dose of −3 had an effect equivalent to a dilution of the virus of at least 1:1000, or that neutralization of 99.9% of the virus occurred. This estimate was based on dose-latent period curves of 4 studies reported(4) and a fifth recently completed.

Data on the influence of injecting normal and immune serum into susceptible chicks prior to their being inoculated with various doses of virus are summarized in Table II. The mortality from visceral lymphomatosis to 201 days of age among chicks that received the normal serum injections was about the same as among chicks that did not receive any serum. The response in all of the three different dosage lots was less than expected on the basis of other inoculation results(4) with the same virus preparation. However, the responses were much greater than obtained with any of the 3 dosage lots in chicks injected with immune serum. Thus, none of the chickens that had received a total of 2 ml of immune serum during the first 3 days of life, and subsequently were inoculated with virus at a dosage of log −5 and log −7, and only 3 of 24 chickens similarly treated and challenged with the maximum dose of log −3, developed vis-

TABLE II. *In Vivo* Neutralization of the Virus of Visceral Lymphomatosis by Specific Antiserum.

Serum	Log dose of virus	No./lot Total	No./lot Corrected	% visceral lymphomatosis at 201 days	Latent period (days)
0	−3	22	20.9	47.8	92.7
	−5	24	22.1	13.6	144.8
	−7	23	22.2	13.6	171.3
Normal	−3	23	20.7	63.8	83.1
	−5	23	21.4	14.0	157.1
	−7	24	23.4	14.0	144.3
Immune	−3	24	23.5	12.7	188.0
	−5	24	23.3	0	
	−7	23	22.9	0	—
Non-inoc. controls		39	38.2	0	—

ceral lymphomatosis. Furthermore, these 3 died late in the experimental period, resulting in a mean latent period greater than that of any other lot.

Estimates of neutralization based on the latent period data of the *in vivo* experiment, are not nearly as reliable as those based on the *in vitro* experiments, because of the small number of birds that died; however, the differences are of such magnitude that they add considerable weight to the quantal response data.

Results from the 2 experiments described demonstrated the presence of an antibody which neutralizes the oncogenic activity of the virus of visceral lymphomatosis. This antibody was found to be present in serum of chickens after they had received a series of injections of filtered extract of lymphomatous liver containing the active virus, and was essentially absent in serum of the same chicks collected just prior to the injection of the virus. These chickens were from a flock that had been reared in isolation for many years and were relatively free from infection with any known disease agent.

There have been many reports [see review of Olson(5), and the reports of Kabat and Furth(6), Furth and Kabat(7)] of indications of immunity and of serum antibodies in experimental work with the leukemic forms of avian leukosis. Recently, Eckert *et al.*(8) working with the virus of myeloblastosis were regularly able to provoke the formation of specific neutralizing and precipitating antibodies in normal growing chickens not previously exposed. This was done by repeatedly administering virus concentrates inactivated by formalin and then injecting highly infectious plasma. Lee(9) reported the development in ducks and turkeys of neutralizing antibodies effective against myeloblastosis and neurolymphomatosis. There is indirect evidence to indicate that the various forms of avian leukosis are caused by different etiologic agents(10-12).

Summary. Evidence is presented for the production of antibodies which neutralize the oncogenic activity of the virus of visceral lymphomatosis, by hens injected with virulent virus. Serum from such hens injected into chicks has produced a passive immunity.

1. Burmester, B. R., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 153.

2. Burmester, B. R., and Cottral, G. E., *Cancer Res.*, 1947, v7, 669.

3. Burmester, B. R., and Gentry, R. F., 91st Ann. Meet. Am. Vet. Med. Assn. Proc., 1954, pp311-316.

4. Burmester, B. R., *Poultry Sci.*, in press.

5. Olson, Carl Jr., *Mass. Agr. Exp. Sta. Bull.* 370.

6. Kabat, E. A., and Furth, J., *J. Exp. Med.*, 1941, v74, 257.

7. Furth, J., and Kabat, E. A., *ibid.*, 1941, v74, 247.

8. Eckert, E. A., Sharp, D. G., Beard, Dorothy, Green, I., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 181.

9. Lee, C. D., *Am. J. Vet. Res.*, 1942, v3, 336.

10. Beard, J. W., Sharp, D. G., and Eckert, E. A., in press.

11. Burmester, B. R., Gentry, R. F., and Waters, N. F., *Poultry Sci.*, 1955, v34, 609.

12. Waters, N. F., *ibid.*, 1954, v33, 365.

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Antigens from Yeast Phase of *Histoplasma capsulatum*. II. Immunologic Properties of Protoplasm vs. Cell Walls. (22012)

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Antigens from the yeast phase of *Histoplasma capsulatum* have been shown to enter into several immunologic or serologic reactions. In complement-fixation tests for detection of antibodies in experimental and naturally occurring histoplasmosis, antigens have included whole cells(1,2) and supernatant fluid from ground cells(3). In precipitation tests, a filtrate from the growth of cells of the yeast phase in broth has been used extensively(4). These complex antigens are of definite value as aids in diagnosis, although some cross-reactions occur with sera from other mycoses, primarily blastomycosis and coccidioidomycosis(5-7). Recently, attempts have been made to improve the specificity of the yeast-phase antigens(8,9). An endotoxin was demonstrated in formalin-killed whole cells of the yeast phase by intraperitoneal injection into 21-day-old white Swiss male mice (10). Inoculation of similarly prepared cells of the yeast phase in smaller quantities induced the development of resistance against subsequent lethal intracerebral or intraperitoneal challenges(11,12). Reactions of tuberculin or delayed type in sensitized individuals after injection of yeast-phase antigens are well known(13). Recently, it was also reported that hypersensitive mice could be killed in less than 48 hours by injection of dead whole cells in 5% mucin(14). Hemolysins that were most active against hen and guinea pig erythrocytes were demonstrated in soluble extracts from the yeast phase(15). Until recently, it had been believed that each cell of the yeast phase was enveloped in a capsule(16). Studies with the electron microscope, however, showed that these cells did not have a capsule(17). Since whole cells had been proven to be useful and active as antigens, the question arose as to where in the cell each antigen was located.

In this paper, methods are described for the separation of cell walls from the protoplasm and data are presented illustrating some of the antigenic properties of these two fractions.

Materials and methods. White Swiss male mice, 21 days old and raised at the Rocky Mountain Laboratory, were employed. One isolate of *H. capsulatum* was used throughout, namely #6515, which originally had been isolated from a dog. It was maintained at 37°C on an agar medium consisting of 51 g/1 Difco cystine heart agar and 21 g/1 Difco bacto-hemoglobin. The yeastlike cells for preparation of antigen and for injection into animals were grown for 3 days under constant rotation at 37°C in a liquid medium containing salts, dextrose, and amino acids(12). The immunologic and serologic tests were similar to those previously described(1,18). *Preparation of Antigens.* Antigens were prepared by one of two methods, both of which included vibration with glass beads (#1241 glass beads, obtained from Cataphote Corp., Toledo, O.) in a container of a Mickle tissue disintegrator (manufactured by H. Mickle Co., Hampton, England). The time necessary for exposure of cells of the yeast phase to vibration was determined with the aid of an electron microscope. Varying concentrations of cells were exposed for varying periods of time in the tissue disintegrator. That time and concentration were used for separation of protoplasm from the cell wall wherein the cells were shattered sufficiently for the protoplasmic contents to escape but not enough for the cell walls themselves to be fragmented. For example, many of the ether-killed cells were suspended in 8 ml quantities (10^{10} cells/ml) with 8 g glass beads and vibrated for 10 minutes at 4°C.

Method 1. A cell suspension of the yeast phase was shaken with approximately 3 volumes of ethyl ether in large separatory funnels. At first, the cells sank to the bottom of

FIG. 1. Water-washed cells of yeast phase of *H. capsulatum*. Magnification 20000 ×.

the aqueous portion of this mixture. However, after several shakings over a period of approximately 24 hours, the cells rose to the water-ether interface. Cells were then harvested, washed by centrifugation in distilled water and reshaken with 3 volumes of ether. When cells of the yeast phase once again rose to the interface, they were washed and treated again in ether. After a third washing in distilled water, the cells were exposed to vibration in the Mickle tissue disintegrator. Subsequent washings by centrifugation in distilled water at 2500 g at 4°C produced two fractions: (a) the cell walls and (b) the supernatant protoplasm. The second fraction was filtered through a fine sintered glass filter to remove possible cell-wall fragments. Both fractions were subsequently lyophylized. *Method 2.* Cell suspensions from individual flasks were pooled and the cells washed by centrifugation in distilled water. Cells were then suspended in 1:5000 merthiolate for 5 days at 37°C, then the cells showed no signs of growth when cultured on Sabouraud's agar slants. The cells were washed in distilled water and exposed to vibration in the Mickle disintegrator. After washing of resulting cell residue and filtration of protoplasm, the two fractions were lyophylized. Similar results were obtained in various immunologic tests described below with fractions prepared by these two methods.

Results. Morphology. The morphology of cells of the yeast phase of *H. capsulatum* was examined under the electron microscope after each step in the fractionation procedure. This examination was of great importance in procedures associated with disruption of cells by mechanical agitation and the separation of disrupted products by centrifugation. Representative pictures are included to illustrate changes in morphology (Fig. 1-4).

Fig. 1 shows the original morphology of the yeastlike cell as seen in preparations of cells suspended in water. A purified cell-wall preparation obtained from ether-killed cells is shown in Fig. 2; the corresponding filtered protoplasm, in Fig. 3. Fig. 4 demonstrates the unfiltered supernatant material after merthiolate-killed cells had been cracked in

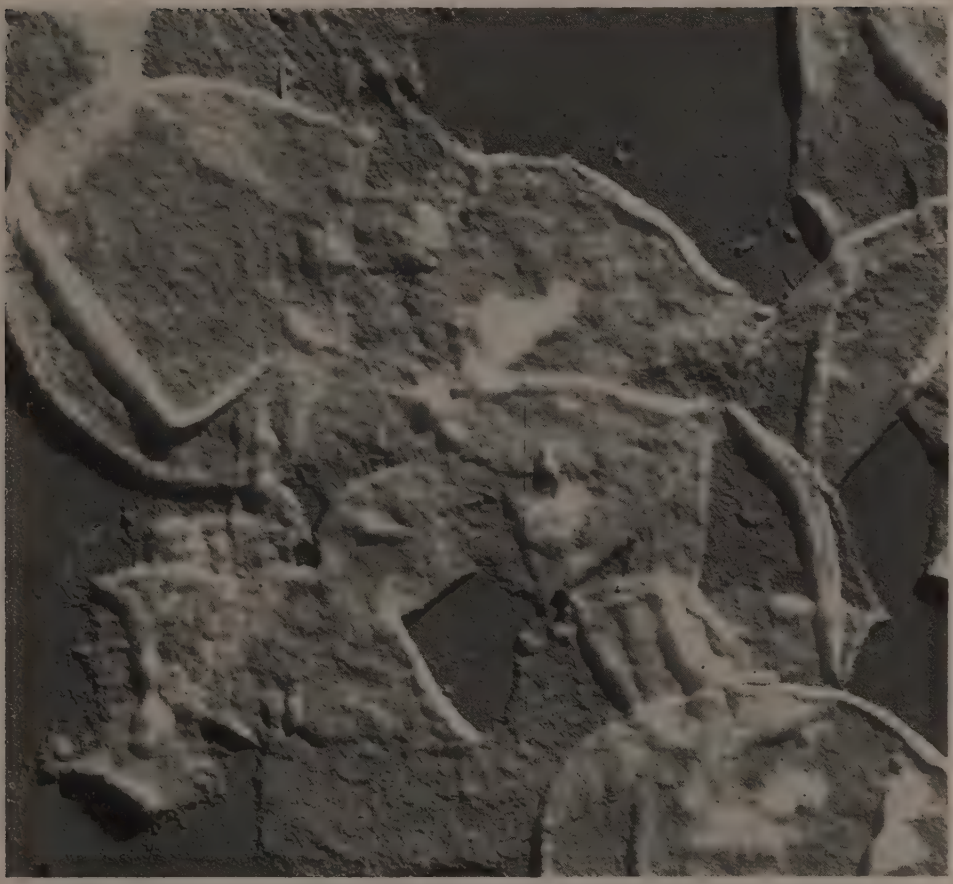

FIG. 2. Cell walls of ether-killed yeastlike cells of *H. capsulatum*. Magnification 44000 ×.

the Mickle disintegrator and centrifuged at 2500 g at 4°C for 30 minutes. Some cell-wall material was invariably present with the soluble protoplasm. The picture demonstrates the striking contrast in morphology between cell wall and protoplasm, and as a result the ease with which the two fractions may be distinguished under the electron microscope.

Examination of the protoplasm in Fig. 3 and 4 indicates that protoplasm of merthiolate-killed cells is released in a very fine, uniform, dispersed state, which condition permits easy separation of cell walls by centrifugation and filtration. In contrast, the protoplasm of ether-killed cells tends to contain more insoluble nondispersible granules, the larger of which are separated from cell walls by centrifugation only with difficulty.

Complement Fixation. The complement-fixing activities of cell walls and protoplasm from ether-killed or merthiolate-killed cells were compared with the activity of heat-killed, lyophilized whole cells in the presence of human sera from proved or suspected cases of histoplasmosis and in the presence of sera from experimentally infected guinea pigs and rabbits. The cell walls had much more complement-fixing activity against human sera than an equal weight of whole cells. The filtered protoplasm, on the other hand, had much less activity than an equal weight of whole cells. The optimum level for whole

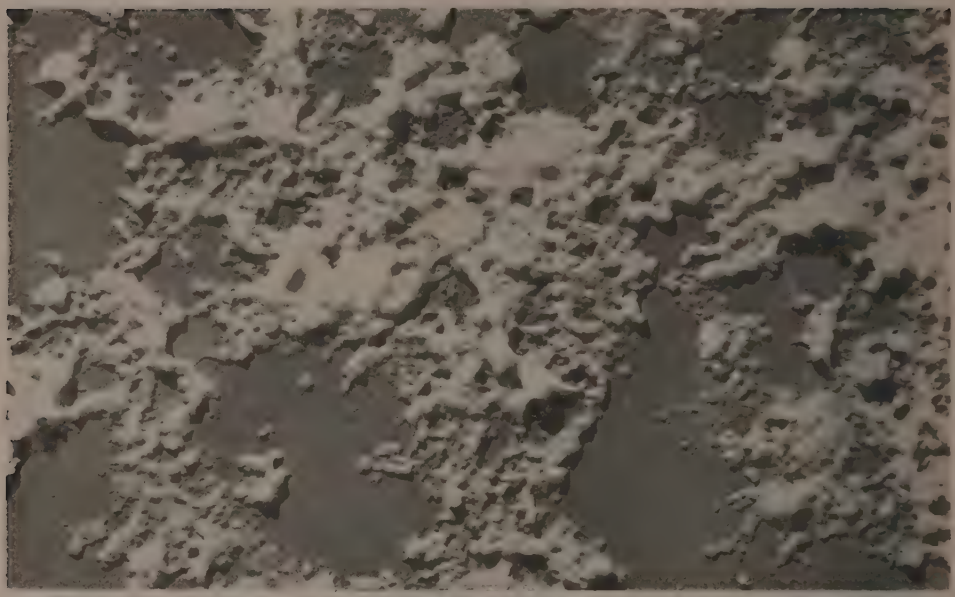

FIG. 3. Filtered protoplasmic fraction from ether-killed yeastlike cells of *H. capsulatum*. Magnification 33000 ×.

FIG. 4. Unfiltered protoplasmic fraction from merthiolate-killed cells of yeast phase of *H. capsulatum*. Magnification 29000 ×.

cells against human or experimentally infected animal sera was approximately 0.5 mg/ml, that for cell walls 0.125 mg/ml, and that for filtered protoplasm 4 to 8 mg/ml.

The possibility existed that some active antigen was washed out of the cell wall into the supernatant liquid in the process of separating the protoplasm. Accordingly, both the sediment and the supernatant fluid were assayed as complement-fixing antigens before initial washing in distilled water, and individually after each successful washing by centrifugation. The results indicated there was a marked increase in complement-fixing powers in both sediment and supernatant liquid as the cell walls were washed free of adherent protoplasm. The increase in activity of the cell-wall fraction may be attributed to gradual separation of the inactive protoplasm from the antigenically active cell wall. The increase in activity of protoplasm was attributed to leaching out of the active complement-fixing antigens from the cell wall during the washing process.

Intraperitoneal injection of the filtered soluble protoplasm or of the cell wall from merthiolate-killed or ether-killed cells induced the formation of complement-fixing antibodies in normal guinea pigs and rabbits, when heat-killed whole cells of the yeast phase were used as complement-fixing antigen. However, cell walls proved to be a much stronger antigen (Table I). When another group of rabbits was similarly injected and subsequently infected with an intravenous dose of 10^7 live cells of *H. capsulatum,* a much greater booster response was noted in the cell-wall fraction as compared with either whole cells or the soluble supernatant liquid (Fig. 5 a and b).

Further exposure of the cell wall to vibration in the Mickle disintegrator resulted in a suspension of minute fragments of cell wall. This suspension, which was clear to the naked eye, was very active antigenically in fixing complement in the presence of sera from cases of human histoplasmosis.

Protection. Mice were protected against lethal intracerebral or intraperitoneal challenge by prior injection of formalin-killed acetone-dried cells of the yeast phase, administered in one or 2 doses. When a single dose was used, 5 mg were administered intraperitoneally and 2 weeks later the animals were challenged. When 2 immunizing doses were employed, 2½ mg injections were given one week apart, and one week after the second dose the mice were challenged. Five mg of either cell-wall material or filtered protoplasm were injected intraperitoneally and the mice subsequently challenged intracerebrally with a lethal dose. In all cases, mice immunized with whole cells and non-immunized mice were included as controls. The protoplasm showed little or no protective powers in contrast to cell-wall material which did have good protective properties. Thus, only 22 of 69 mice (32%) died when first injected with cell walls, and subsequently challenged, in contrast to 53 of 60 mice (89%) that died when first inoculated with the protoplasm and subsequently challenged. Only 32 of 90 mice (35%) immunized with whole cells succumbed to the lethal challenge, while 87 of 93 (93%) of controls died. The cell wall was effective in quantities of approximately 0.5 to 5.0 mg of dried material per mouse, while the optimum dose for whole cells was approximately 5 mg per mouse.

TABLE I. Complement-Fixation Titers of Sera from 40 Rabbits Injected with 30 mg of Cell Walls, Protoplasm, or Whole Cells of *H. capsulatum.**

	Protoplasm	Cell walls	Whole cells	Controls
Range	0–16	128–2048	16–64	0
Median	4	256	32	0
Mode	0	256–512	32	0

* Heat-killed whole cells of the yeast phase were used as the complement-fixing antigen. Ten rabbits were included in each of 4 groups.

Toxin. The toxic action of protoplasm and cell walls was examined in mice, with 2 mg of dried tubercle bacilli incorporated into each mouse dose. When 5 mg or 10 mg of antigen were injected per mouse (Table II), most of the deaths within 48 hours occurred as a result of the action of the cell walls and not of the soluble protoplasm. None of 160 mice receiving adjuvant alone died.

Hypersensitivity. Mice were inoculated intraperitoneally with sublethal dose of 10^6 72 hour cells of the yeast phase. Fourteen days later, these mice were challenged with equal

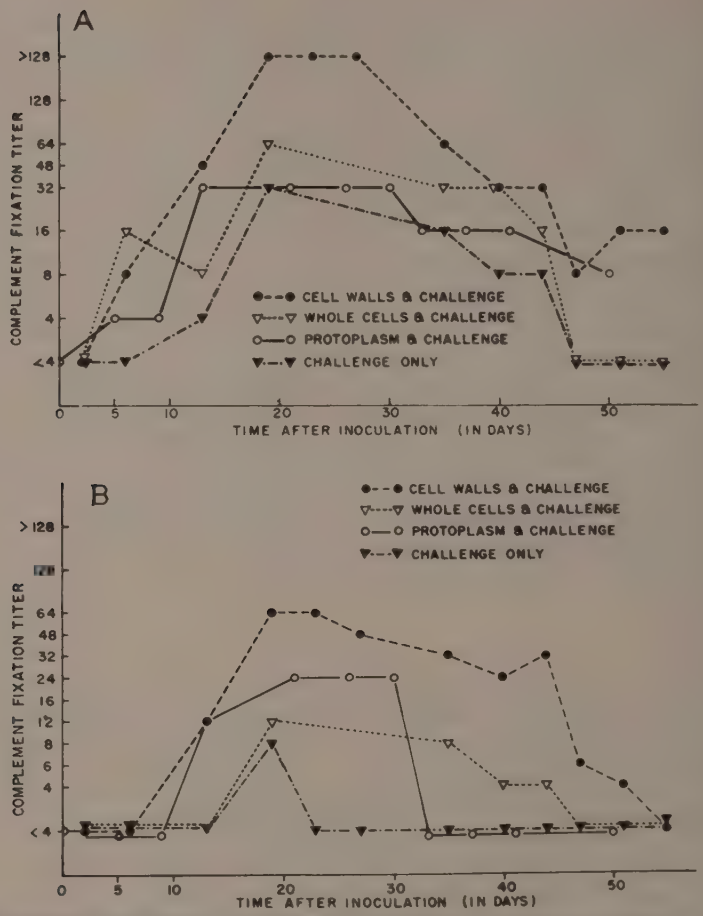

FIG. 5. Influence of cell walls or protoplasm on development of complement-fixing antibodies in experimentally infected rabbits. A. Peak titers. B. Median titers.

weights of whole cells, cell walls, or protoplasm, suspended in 5% mucin. Death rates were high and similar in mice that had been inoculated with cell-wall material or protoplasm during 48 hours post challenge. Either of these fractions produced a higher mortality than whole cells. For example, when 7.5 mg in 5% mucin were injected intraperitoneally per mouse, 40 of 50 mice (80%) succumbed to cell walls, 31 of 35 (89%) to protoplasm, and 12 of 50 (24%) to whole cells. Five of 148 mice (3%) sensitized but not further inoculated, died during this period. None of 50 sensitized mice succumbed after intraperitoneal inoculation of 5% mucin alone. Antigen, or antigens, active in initiating a response in sensitized mice seems to be present in both cell wall and protoplasm. Thirty-five guinea pigs which had been infected with a sublethal dose of the yeast phase were subsequently skin tested with the fractions previously described, as well as with histoplasmin. Edema developed 24 to 48 hours after intradermal injection, both with the cell-wall fraction and with filtered protoplasm. When the fractions were diluted to a similar extent, a slightly greater response was initiated by the soluble protoplasm. Five uninfected guinea pigs showed

TABLE II. Comparison of Toxic Action of Protoplasm and Cell Walls in 21-Day-Old Male Mice.

	Mortality			
	Protoplasm	Cell walls	Whole cells	Adjuvant controls
5 mg	2/70* (3%)	11/70 (16%)	3/70 (4%)	0/70 (0%)
10	10/90 (11%)	55/90 (61%)	16/90 (18%)	0/90 (0%)

* Numerator represents No. of mice that died; denominator represents total No. of mice inoculated. These figures are totals from 3 separate experiments.

no skin reactions at all. Results in sensitized guinea pigs were, therefore, similar to those obtained in sensitized mice.

Hemolysis. A 0.5% suspension of guinea pig erythrocytes in 0.85% saline underwent hemolysis when exposed to 0.125 to 0.25 mg whole cells/ml. Protoplasm showed about as much activity over a similar weight range. The cell wall in comparison showed little hemolytic activity with concentration as high as 8 mg/ml not producing complete hemolysis.

Discussion. With the methods of preparation used in the foregoing experiments, the cell wall of the yeastlike cells of *H. capsulatum* contained most of the antigenic activity responsible for the fixation of complement in the presence of specific antiserum. This fixation of complement occurred with human sera from proven or suspected cases of histoplasmosis and with animal sera from experimentally infected rabbits or guinea pigs. The antigen associated with complement fixation is soluble in water, since extensive washing caused it to pass gradually into the supernatant liquid. Attempts, therefore, to prepare a soluble, purified, specific antigen from the yeast-like cells should be directed toward the cell wall.

The cell wall can be readily separated from the internal protoplasm by vibration and subsequent centrifugation. Mechanical agitation with the Mickle disintegrator apparently cracked the cell walls and released the protoplasm in a dispersed state. The products of disruption, cell walls and protoplasm, were then readily separated by centrifugation. The amount and type of vibration should be carefully controlled, in combination with electron-microscope examinations, in order to permit ready separation of the two fractions. If the cells are exposed to excessive vibration or grinding, then the resulting cell-wall fragments are too small to separate from the coarse granular protoplasm. Cell disintegrators like the Raytheon ultra-sonic oscillator were accordingly found not to be useful, since they tended to fragment the cell wall excessively rather than merely to crack it(19), and, therefore, to make impossible the recovery of purified cell-wall fractions. Excessive ether or merthiolate treatment tended to coagulate the protoplasm and prevent its release in a dispersed state, to such an extent that separation of the protoplasm by washing and centrifugation became impossible.

Similarly, most of the activity responsible for the protection of mice or the toxic death of mice was situated in the cell wall. The identity of or relationship between these 3 antigenic actions is still in doubt. It is of interest, however, to note that complement-fixing antibodies were not readily detected in immunized mice which had marked resistance to reinfection or to lethal challenge. Rabbits and guinea pigs, on the other hand, readily developed complement-fixing antibodies in high titers, but showed little tendency to develop marked resistance to lethal challenge (19).

A delayed reaction in sensitized animals was produced by both cell walls and protoplasm. This result may mean that parts of either fraction are capable of initiating a response, or that the particular antigen involved is highly soluble and readily dissolves out of the cell wall into the surrounding fluid.

Since the tissue phase of *H. capsulatum* is yeastlike in some of its properties, the possibility has presented itself that zymosan may be present in the cell wall(20). If zymosan is present, then it would tend to reduce the bactericidal, and possibly mycocidal, properties of serum. However, the cell walls of *H. capsulatum* make the mice more resistant to subsequent challenge, rather than less resistant. Hence, the zymosan-properdin system, if present, has no obvious influence on the immunization of mice against *H. capsulatum*.

Summary. A technic, involving the use of

the Mickle tissue disintegrator and the electron microscope, was described for the separation of the cell wall and the internal protoplasm from the cells of the yeast phase of *Histoplasma capsulatum*. The cell wall contained the antigenic activity associated with complement fixation, protection and toxins. Sensitized animals, on the other hand, showed a delayed type of reaction to either fraction. The protoplasm was more active than the cell wall in the hemolysis of guinea pig erythrocytes.

1. Salvin, S. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 342.

2. Grayston, J. T., *J. Lab. and Clin. Med.*, 1952, v40, 90.

3. Saslaw, S., and Campbell, C. C., *ibid.*, 1948, v33, 1207.

4. Salvin, S. B., and Hottle, G. A., *J. Immunol.*, 1948, v60, 57.

5. Salvin, S. B., *J. Lab. and Clin. Med.*, 1949, v34, 1096.

6. ———, *J. Immunol.*, 1950, v65, 617.

7. Campbell, C. C., and Binkley, G. E., *J. Lab. and Clin. Med.*, 1954, v42, 896.

8. Campbell, C. C., *Am. J. Pub. Health*, 1953, v43, 712.

9. Sorenson, L. J., and Evans, E. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 339.

10. Salvin, S. B., *J. Immunol.*, 1952, v69, 89.

11. ———, *ibid.*, 1953, v70, 267.

12. ———, *Am. J. Hyg.*, 1955, v61, 72.

13. Zarafonetis, C. J. D., and Lindberg, R. B., *Univ. Hosp. Bull.*, Ann Arbor, 1941, v7, 47.

14. Salvin, S. B., *J. Immunol.*, 1955, in press.

15. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 852.

16. Skinner, C. E., Emmons, C. W., and Tsuchiya, H. M., *Molds, Yeasts, and Actinomycetes*. John Wiley and Sons, N. Y., 1947.

17. Ribi, E., and Salvin, S. B., *Experimental Cell Research* (in press).

18. Salvin, S. B., and Furcolow, M. L., *J. Lab. and Clin. Med.*, 1954, v43, 259.

19. Salvin, S. B., and Ribi, E., unpublished results.

20. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science*, 1954, v120, 279.

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Reproduction in Rats Fed *Lathyrus* Peas or Aminonitriles. (22013)

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Growth disturbances produced by feeding *Lathyrus* peas vary inversely with age and stage of development of the experimental animal(1,2). Profound effects might be expected, therefore, during rapid growth and development of the fetus. Turney, Salmon, and Copeland reported that feeding *Lathyrus hirsutus* peas prevented completion of pregnancy in the rat, but they did not completely describe the nature of the disturbance(3). They were unable to prevent the effect of peas by addition of alpha-tocopherol to the diet(4), although it has since been reported that large doses of that compound give partial protection against lathyrism(5). For these reasons the effect of *Lathyrus odoratus* peas was studied in regard to reproduction. During this investigation, isolation of the active principle of *Lathyrus pusillus* was announced(6). A similar isolation product of *Lathyrus odoratus* was identified as (β-L-glutamyl-amino) propionitrile(7). The characteristic activity in producing skeletal lesions in rats was also shown by β-amino-propionitrile(1). This compound also profoundly influenced development of larval frogs(2). Aminoacetonitrile was later found to be more toxic to weanling rats than any compound previously tested(8). A number of aminonitrile compounds were consequently included in this study.

Methods. Albino rats of Sprague-Dawley strain were used predominantly. The few hooded rats of Long-Evans strain used gave identical results. The basic diet was ground (Rockland) rat pellets. *Lathyrus odoratus* seeds were finely ground, mixed with this diet in various proportions, refrigerated at 5°C, and fed *ad libitum*. Diets containing syn-

thetic nitrile compounds were stored at –40°F. Feedings were given once daily, and food remaining after 24 hours discarded. All animals were kept on wire-mesh floors. The 50% pea diet produced skeletal deformities and fatal aortic rupture comparable to that reported by Ponseti and Baird(9). Females were first mated when 10-12 weeks of age, and discarded after 3 or 4 matings. Matings were determined by vaginal smear. Specimens for histological examination were fixed in 4% formalin and stained with hematoxylin and eosin.

Results. Mature rats of both sexes maintained on 50% pea diet remained in good health for several months. Two male rats placed on this regimen at 6 weeks of age developed extensive skeletal deformities within a few weeks, but accomplished numerous fertile matings between 3 and 9 months. When sacrificed at 10 months they were moderately emaciated and almost helplessly crippled, but showed active spermatogenesis, without testicular atrophy. A third male, started on 50% pea diet at 3 months, was moderately crippled but still potent and fertile when sacrificed 10 months later. This male also showed histologically normal testes. The numerous offspring obtained by mating these males with normal females showed no abnormalities.

The 50% pea diet given to nursing female rats produced no evident toxic effect. In one case this diet was given to the dam of 6 young, beginning on the 3rd day after parturition. In 2 instances this diet was begun on the 20th day of pregnancy, and continued until litters were weaned. Large litters were born, of which 3 in one litter and 5 in the other survived the early neo-natal period. The high peri-natal mortality was typical of results obtained when pea diets were given late in pregnancy, and will be discussed in detail later. The nurslings of all 3 litters made normal weight gains, with no physical or x-ray evidence of skeletal deformity at weaning at 22 days. Three males and 3 females given 50% pea diet after weaning quickly developed severe skeletal deformities, and 2 animals of each sex died of ruptured aortic aneurisms within a month. Those given control diet grew to normal maturity.

Mature female rats on 50% pea diet passed through normal estrous cycles, and mated freely. Conception occurred in a high percentage of matings, with bloody vaginal "placental sign" on about the 13th day. Weight gains were comparable to control animals until the 19th or 20th day, but then weight rapidly decreased, and no living young were born. Pregnant females sacrificed on or before 19th day of pregnancy contained living fetuses, but during the succeeding 3 days there was death and rapid decomposition of all unborn young. Passage of dead fetuses or placental tissue was seldom observed, although vaginal bleeding was often seen at the 22nd or 23rd day. Animals killed within a few days after this time showed involuting uteri with numerous placental sites. Those kept on 50% pea meal soon displayed normal estrous cycles culminating in fertile matings. If the control diet was then given, normal gestations resulted.

TABLE I. Effect of 50% Pea Meal.

No. rats	Days of gestation diet given	Avg No. viable young
9	1-22	0
4	1-16	8.5
6	1-17	.67
4	16-18	.25
6	19-22	0
7	20-22	4
4	21-22	12

The final few days of gestation constituted the critical period for exhibition of the toxic effect of the pea (Table I). Normal litters were born to females on 50% pea diet during the first 16 days of pregnancy, but giving this diet for one more day resulted in death of almost all fetuses. Giving the diet for as short a time as 3 days after the 16th day was also highly toxic for the unborn young.

Diets containing 20% *Lathyrus odoratus* meal were lethal for the rat fetus, if begun by the 17th day of gestation (Table II). The

TABLE II. Percentage of Lathyrus Pea Meal Begun 17th Day of Gestation.

No. rats	% pea meal	Avg No. viable young
9	50	0
8	20	.5
2	15	2
3	10	10

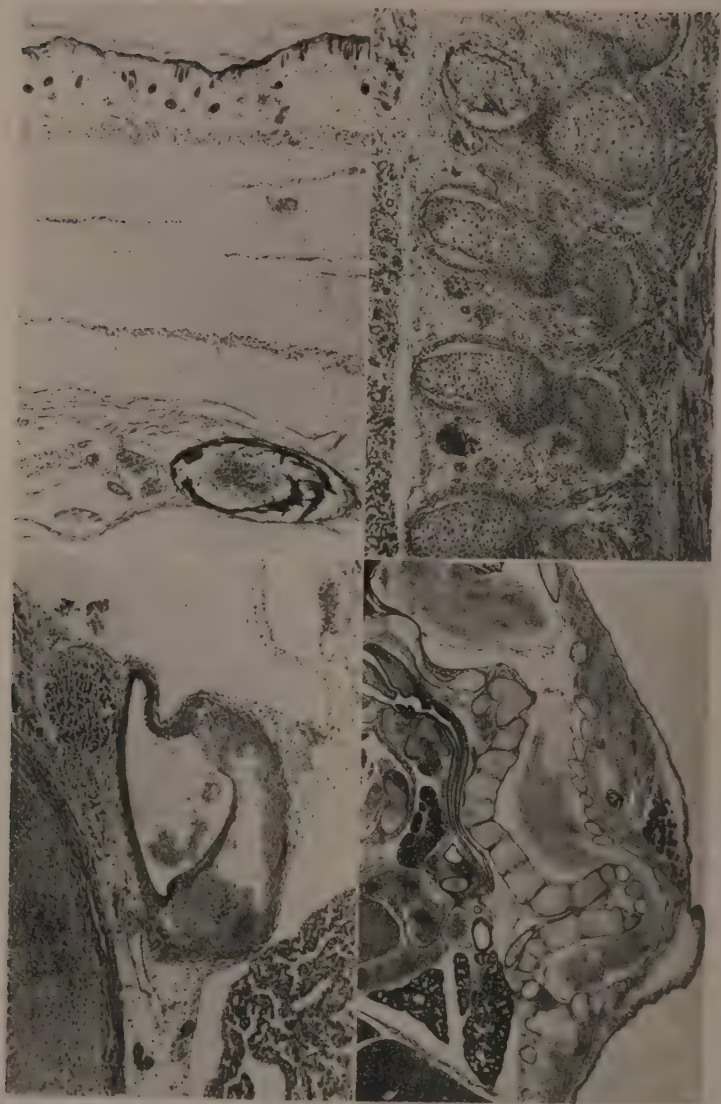

FIG. 1 (top left). Microphotograph, posterior thorax of still-born rat. Includes tissues from skin surface to parietal pleura, with cross section of rib, lower right. There is extreme generalized edema, with paucity of connective tissue and skeletal muscle.

FIG. 2 (top right). Microphotograph, parasagittal section, thoracic spine of 19-day rat fetus, alive at time of sacrifice of dam, who had received 50% pea diet from first day of pregnancy. Note lack of cohesion between cartilaginous spinal structures and perichondrial connective tissue.

FIG. 3 (bottom left). Microphotograph, cross section of dissecting aneurism, thoracic aorta of still-born rat whose dam received 50% pea diet from 17th day of pregnancy. The intima is ruptured and a large mass of blood has forced apart the layers of the media. Complete rupture of the vessel, with massive hemothorax, has not taken place in this case.

FIG. 4 (bottom right). Microphotograph, mid-sagittal section, 19-day rat fetus, litter-mate of fetus shown in Fig. 2. There is acute angulation of the lower thoracic and lumbar spine, with apparent compression of the spinal cord. Diffuse hemorrhage is seen, particularly about great vessels at base of heart. The fetus was alive at time of sacrifice of the dam.

TABLE III. Toxicity of Aminonitriles Begun 17th Day of Gestation.

Compound	% in diet	No. rats	Avg No. viable young
Aminoacetonitrile*	.0167	4	0
	.0125	10	3.1
β-aminopropionitrile	.3	3	0
	.1	9	0
	.05	6	0
	.025	9	6.2
Bis (β-cyanoethyl)amine	.1	3	0
	.05	2	10
Tris (β-cyanoethyl)amine	.3	3	8
β-dimethylaminopropionitrile	.3	2	9
β-mercaptoethylamine	.5	5	3.4
	.25	3	5.7

* Fed as a salt of H_2SO_4.

15% level was incompletely lethal, and lesser concentrations apparently harmless. At 20% level many young were born dead, with little evidence of decomposition, while others were feebly alive at birth, but succumbed soon thereafter. Both living and dead were fragile and hydropic in character (Fig. 1). Muscles and connective tissues were poorly developed. There was scanty formation of collagen, and cohesion of various skeletal and connective tissues appeared faulty (Fig. 2). Poor vascular tone, with capillo-venous dilatation and diffuse hemorrhage was commonly seen. Massive hemothorax from aortic rupture was a frequent demonstrable cause of death (Fig. 3). The dissecting aortic aneurisms followed the same pattern seen in weanling rats(1,9). Severe spinal deformities seen in several instances included kypho-scoliosis similar to those typically produced in weanling rats on *Lathyrus* pea diets (Fig. 4).

Several nitrile compounds were toxic for the rat fetus (Table III). Their effect appeared to be qualitatively identical with that of pea diets. Fetal death was readily produced by feeding diets containing β-aminopropionitrile, bis (β-cyanoethyl) amine, or aminoacetonitrile during the latter part of pregnancy. Aminoacetonitrile was the most toxic of these compounds, as shown by Wawzonek and coworkers(8). Lesions produced by these compounds mimicked those produced by pea meal diets, including a high incidence of aortic rupture with hemothorax. As with pea meal diets, little toxicity was shown during the first 16 days of gestation. Thus, normal litters were born to female rats fed a diet containing 0.1% β-aminopropionitrile during the first 16 days of pregnancy, whereas diets with 0.05% of this compound proved uniformly lethal to fetuses when given beginning the 17th day of pregnancy (Table III). It is perhaps significant that the obvious developmental defects produced involved mesodermal tissues, and that development of these lesions occurred during the period of rapid mesodermal growth and differentiation of late fetal life. A possible difference in placental permeability in regard to the toxic compounds might also account for differences in toxicity shown during early and late fetal life.

Two nitrile compounds tested, tris (β-cyanoethyl) amine and β-dimethylaminopropionitrile, showed no toxicity at concentrations used. These results are in accord with previous reports of the detoxifying effect of methylation or acetylation of aminonitrile compounds(10). The final compound listed in Table III, β-mercaptoethylamine, while not an aminonitrile, has produced skeletal growth disturbances typical of lathyrism(11). This compound was found to be relatively innocuous for the pregnant rat and her unborn young, although at comparatively high concentrations many fetuses did die *in utero*. None of these fetuses, however, showed skeletal deformity, ruptured aortae, or other lesions characteristic of lathyrism.

Summary. *Lathyrus odoratus* peas and certain aminonitrile compounds chemically related to the active extract of the pea, when added to the diet of pregnant rats, caused death of the fetal rat late in intrauterine life. Death was associated with poor development of the skeleton and other mesodermal tissues, and commonly resulted from rupture of the thoracic aorta at or near the time of birth. No harmful effects on the fetus were demonstrated by feeding these substances to the pregnant female before the 17th day of gestation. *Lathyrus odoratus* peas in 50% dietary concentration did not affect fertility of the adult male or female rat, and males maintained on the diet for as long as 10 months showed no histological evidence of testicular atrophy.

No toxic substance was demonstrated in the milk of nursing females on the pea diet. The feeding procedure employing pregnant female rats proved to be a reasonably accurate and sensitive method for the toxicity assay of aminonitriles. Aminoacetonitrile was the most toxic of the compounds tested.

1. Ponseti, I. V., and Shepard, R. S., *J. Bone and Joint Surg.*, 1954, v36-A, 1031.

2. Chang, C. Y., Witschi, E., and Ponseti, I. V., *Anat. Rec.*, 1954, v120, 816.

3. Turney, D. M., Copeland, D. H., and Salmon, W. D., *Ala. Agri. Exper. Sta. Ann. Rep.*, 1943-44, v54-55, 18.

4. Turney, D. M., Salmon, W. D., and Copeland, D. H., *ibid.*, 1945-46, v56-57, 18.

5. Lee, J. G., *J. Nutrition*, 1950, v40, 587.

6. Dupuy, H. P., and Lee, J. G., *J. Am. Pharm. A*, (Scient. Ed.), 1954, v43, 61.

7. Schilling, E. D., and Strong, F. M., *J. Am. Chem. Soc.*, 1954, v76, 2848.

8. Wawzonek, S., Ponseti, I. V., Shepard, R. C., and Wiedenman, L. G., *Science*, 1955, v121, 63.

9. Ponseti, I. V., and Baird, W. A., *Am. J. Path.*, 1952, v28, 1059.

10. Bachhuber, T. E., Lalich, J. J., Angevine, D. M., Schilling, E. D., and Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 294.

11. Dasler, W., *ibid.*, 1955, v88, 196.

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Effect of Diet on Betaine-Homocysteine Transmethylase of Rat Liver.* III. B-Vitamins Other Than Vitamin B_{12}. (22014)

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In the rat, a dietary deficiency of vit. B_{12} causes a decrease in the betaine-homocysteine transmethylase activity of the liver(1-4). The addition of a purified "coenzyme" preparation of the transmethylase, to liver homogenates prepared from rats fed a diet deficient in vit. B_{12}, has recently been shown to restore the transmethylase activity(4). To ascertain whether this effect is specific for vit. B_{12} the effect of a deficiency of each of the other B-vitamins on the transmethylase activity of rat liver has been investigated.

Methods. Albino rats of the Sprague-Dawley strain were used throughout the investigation. The rats were kept in individual cages and were fed *ad libitum*, unless otherwise stated. The complete vitamin mixture used for the various basal diets described below provided in mg per 100 g of ration: thiamine hydrochloride, 0.5; riboflavin, 0.5; niacin, 1; calcium pantothenate, 2; pyridoxine, 0.25; folic acid, 0.02; biotin, 0.01; vit. B_{12}, 0.002; inositol, 10; and choline chloride, 150. Each week each rat was given orally 2 drops of halibut liver oil fortified with vit. E and K (5). For the deficient diets the vitamin under study was omitted and in some cases succinyl sulfathiazole ("sulfasuxidine") or the corresponding antivitamin was added to the diet as described in the tables of results. All diets contained 4% of salts 4(6) and 5% of corn oil. The basal diets contained the following percentages of protein, sucrose and supplementary amino acids: Diet I. Casein 25, sucrose 65.8. Diet II. Casein 25, DL-methionine 0.2, sucrose 65.6. Diet III. Casein 12, DL-methionine 0.2, DL-threonine 0.36, sucrose 78.2. Diet IV. Egg white 24, sucrose 66.8, the biotin content of the complete diet was increased to 0.1 mg per 100 g of ration. Diet V. Casein 9, gelatin 12, sucrose 69.8.

At the end of the experimental period the rats were killed by decapitation and were bled, and their livers were removed immediately and chilled in ice. Pooled homogenates

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TABLE I. Effect of Vitamin Deficiencies on Activity of Betaine-Homocysteine Transmethylase of Rat Liver.

	Exp. I					Exp. II				
			Rate of gain	Transmethylase				Rate of gain	Transmethylase	
Vitamin	Diet*	Duration of exp., days	Control, g/wk	Deficient, g/wk	Control, μg	Deficient, μg	Diet*	Duration of exp., days	Control, g/wk	Deficient, g/wk	Control, μg	Deficient, μg
Thiamine	I	21	41.3	9.8	410	450	II	10	33.6	24.5	445	595
Riboflavin	I	14	35.0	19.5	320	460	II	10	33.6	24.2	445	550
Niacin	I	21	38.8	37.1	360	310	III	9	23.3	10.1	400	445
Pyridoxine	I†	14	52.0	5.0	405	535	II	10	33.6	14.0	445	595
Pantothenic acid	I‡	18	38.8	17.8	220	270	II	10	33.6	24.5	445	550
Biotin	I‡	21	40.0	35.0	360	380	IV	24	30.1	23.3	635	490
Choline	V§	14	21.0	15.0	575	535	V	9	21.0	9.1	385	365

* Diets are described in the experimental section. Weanling rats were used throughout unless otherwise noted.
† This diet contained 3 mg % desoxypyridoxine. Initial wt of rats was approximately 85 g.
‡ This diet contained 2% sulfasuxidine.
§ Initial wt of rats was approximately 150 g.

were prepared from the livers of 5 or 6 rats from each experimental group and the transmethylase activity was determined as described previously(4). The transmethylase activity is expressed as μg of methionine formed per g of fresh liver during a 3 hour incubation period.

Results. The effect of a deficiency of each of the following: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin and choline, on the activity of the betaine-homocysteine transmethylase of rat liver is shown in Table I. It can be seen that the transmethylase activity of the livers of the rats fed the deficient diets was generally *higher* than that of the control group. Only in the case of a biotin (Exp. II) or a choline deficiency was the transmethylase activity found to be lower than that of the corresponding control group. The effect of the biotin deficiency on the transmethylase was evident only when the deficiency was severe enough to retard the growth of the rats significantly (compare rate of gain and transmethylase activity in Exp. I and II) and even then the absolute value for the deficient group was quite high. Omission of choline from the (high protein) diet used in these experiments lowered the transmethylase activity only slightly; however, the results are in agreement with previous findings with low protein diets(7).

The results of 5 experiments with rats made deficient in folic acid in various ways are given in Table II. The transmethylase activity of livers of deficient rats was in each case higher than in the corresponding control group. This is particularly noticeable in experiments in which no antivitamin (Aminopterin, Lederle Laboratories, Pearl River, N. Y.) was administered. Experiments with pair-fed rats (Exp. II) demonstrated that high transmethylase activity of deficient animals could not be attributed to an effect of starvation(7). Livers from rats fed diet deficient in both folic acid and vit. B_{12} (Exp. III) also had a higher transmethylase activity than livers of rats fed the complete diet. As the diet in this case contained casein, and growth was retarded by development of a folic acid deficiency, it is doubtful whether a deficiency of vit. B_{12} developed. Dinning *et al.*(8) have

TABLE II. Effect of Folic Acid Deficiency on Activity of Betaine-Homocysteine Transmethylase of Rat Liver.

Exp. No.	Treatment	Duration of exp., days	Initial wt of rats, g	Rate of gain Control, g/wk	Rate of gain Deficient, g/wk	Transmethylase Control, μg	Transmethylase Deficient, μg
1	Diet I + 2% sulfasuxidine	21	45	40.0	25.0	360	685
2	" I + 2% sulfa (pair fed)	21	45	25.2	18.5	380	800
3	" I + 2% sulfa—B_{12}	21	45	35.6	21.0	365	840
4	" I + 4 mg/kg aminopterin	7	150	−23.0	−23.0	445	505
5	" II + 1 " "	7	210	20.0	15.0	475	485

reported that, in chicks, a dietary deficiency of folic acid causes a decrease in betaine homocysteine transmethylase activity of the liver. Williams(9) found liver transmethylase activity of rats fed a complete diet containing aminopterin (4 mg/kg of diet) and showing very severe symptoms, to be lower than that of comparable group receiving complete diet without aminopterin. It is difficult to compare our results with these results since in our investigation, comparison was made between a group that received a complete diet and one that received a folic acid-deficient diet, both of which received aminopterin. It is of interest that Kring and Williams(10) found the activity of liver tyrosine oxidase system, higher in rats receiving diets deficient in thiamine than in control rats receiving an adequate diet, and that Beaten and Ozawa(11) found the activity of glutaminase I to be increased in vit. B_6-deficient rats. There are also several reports (see reference 10) that folic acid deficiency causes increased xanthine oxidase activity of rat liver.

A comparison of values for growth and for transmethylase activity of livers of rats fed Diet I (containing 25% casein) and Diet II (Diet I, supplemented with 0.2% DL-methionine) shows that a supplement of sulfur amino acids stimulated synthesis of betaine-homocysteine transmethylase although no growth response was observed.

The experiments do not provide any clue as to the nature of the "coenzyme" of betaine-homocysteine transmethylase. However, the data presented here and elsewhere(4,7), give some insight into the extremely complex relationships that apparently exist between dietary factors and activity of this enzyme.

Summary. When a diet deficient in one of the following vitamins: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, or folic acid was fed to rats, the activity of the betaine-homocysteine transmethylase of their livers was found to be higher than that of the livers of rats fed a complete diet. Deficiencies of biotin or choline, on the other hand, caused a slight decrease in the transmethylase activity.

1. Oginsky, E. L., *Arch. Biochem.*, 1950, v26, 327.
2. Williams, J. N., Jr., Monson, W. J., Sreenivasan, A., Dietrich, L. S., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1953, v202, 151.
3. Mistry, S. P., Vadopalaite, I., Chang, I., Firth, J., and Johnson, B. C., *ibid.*, 1955, v212, 713.
4. Ericson, L.-E., Harper, A. E., Williams, J. N., Jr., and Elvehjem, C. A., *ibid.*, in press.
5. Harper, A. E., Benton, D. A., Winje, M. E., Monson, W. J., and Elvehjem, C. A., *ibid.*, 1954, v209, 165.
6. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *ibid.*, 1941, v138, 459.
7. Ericson, L.-E., and Harper, A. E., *ibid.*, in press.
8. Dinning, J. S., Keith, C. K., and Day, P. L., *ibid.*, 1951, v189, 515.
9. Williams, J. N., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 206.
10. Kring, J. P., and Williams, J. N., Jr., *ibid.*, 1954, v87, 97.
11. Beaton, J. R., and Ozawa, G., *J. Biol. Chem.*, 1955, v214, 685

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Transmission of Street Rabies Virus Strain (V308) to Suckling Hamsters During Lactation. (22015)

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It has been assumed that a bitch infected with rabies transmitted the disease to the puppies by injuring them by biting or other mechanical injuries. There is still the question as to whether or not rabies virus may be present in the milk and if so whether the puppies can become infected from this source. Specific inclusion bodies called Negri bodies (1) are present in the central nervous system of animals dying with rabies. These are most frequently demonstrable within the nerve cells and are eosinophilic bodies, usually spherical. 1 to 30 μ in size. Their inner structure shows basophilic granules. The Syrian hamster infected with rabies virus shows Negri bodies in the brain in greater numbers and of larger size than those demonstrable in the Swiss albino mouse(2). The Syrian hamster was, therefore, selected for these experiments not only because of its Negri body "production" but because of ease in control and care.

Materials and methods. The virus for the present study was obtained from a dog brain sent to this laboratory for routine rabies examination. The hippocampus was removed aseptically, ground with alundum in a mortar, and diluted with physiological saline to a 10% suspension. Three-hundredths ml of this 10% suspension was injected intracerebrally into each of ten 3-week-old Swiss albino mice (Carworth Farm, Webster strain). On the 14th day after injection, several mice developed tremors and paralysis and became moribund. These brains were then removed aseptically. Impressions made from the Ammons horn of each mouse were stained with Seller's stain(3) and examination by the light optical microscope showed numerous Negri bodies throughout the tissue. The Ammons horn from one of these mice contained a larger number of Negri bodies than the others and it was ground with alundum and diluted to a 20% suspension with physiological saline. Each of ten 3-week-old Swiss albino mice was injected intracerebrally with this rich virus suspension. Titer of the virus was $10^{-3.5}$ when titrated intracerebrally in Swiss albino mice of the same age. The 10 Swiss albino mice which were infected intracerebrally showed rabies symptoms within 6 to 10 days. Their brains which were removed aspetically contained Negri bodies and were ground and diluted to a 10% suspension in physiological saline. This virus-bearing mouse brain suspension was used to initiate the present study. For this series of experiments, diagnosis of rabies in hamsters and mice was made by the following criteria: (1) clinical observations of each hamster, (2) demonstration of Negri bodies in the Ammons horn of the suckling hamster, adult hamster and Swiss albino mouse, (3) reinoculation of the positive and negative suckling hamster brain and uninoculated controls intracerebrally into 3-week-old Swiss albino mice and confirmation by demonstration of Negri bodies in the brain smears. The presence of Negri bodies was demonstrated microscopically in touch preparations of the Ammons horn stained with Seller's stain. Ten lactating hamsters and their sucklings were placed in individual metal boxes with bedding. The sucklings were about 12 days old and are usually weaned at 16 to 18 days. Therefore, these were removed from the parent 6 days after her inoculation. The lactating hamster from each group (#1 to #9) was given 0.1 ml of the above described mouse brain bearing virus via the flank muscle. The area of injection was sterilized with iodine for one hour before the mother from each of the 9 groups was placed with her sucklings. The No. 10 group was kept as a room control. During the next 6 days, the family of each group was not disturbed. Dog checkers were fed to each of the 10 groups daily along with a large bottle of water for drinking (Table I). At the end of the sixth day, after inoculation of the parent, the sucklings from each group were weaned and placed in special glass jars. The lactating hamsters showed no rabies

symptoms at this time and were held for further observation. Seven of the 9 inoculated lactating adults developed rabies symptoms between the 14th and 22nd day after inoculation. Their brains contained numerous Negri bodies. The 10 groups of weaned hamsters from the 9 infected lactating animals and the control hamster were fed, watered and checked twice daily (Table II) for rabies symptoms.

Results. Two days after weaning or 8 days after injection of the adults, several hamsters from Groups 1, 2 and 3 (Table II) showed rabies symptoms and their brains contained numerous Negri bodies. Brains from all suckling hamsters showing rabid symptoms were ground in a mortar with physiological saline to a 20% suspension and injected intracerebrally into each of 6 Swiss albino mice. Mice showing rabies symptoms were examined for Negri bodies as shown in Table II. At the end of 21 days all suckling hamsters showing no rabies symptoms were sacrificed and their brains were removed aseptically and pooled for each group, ground in a mortar with alundum and diluted to a 20% suspension with physiological saline. Each of the 10 pooled groups, including the control Group #10, were injected (0.03 ml each mouse) intracerebrally in each of 6 Swiss albino mice (Table II) in order to confirm the presence or absence of the virus in sucklings showing no rabies symptoms.

Summary. From this observation it appears that rabies may be transmitted from lactating hamsters to suckling hamsters. Seventy-seven percent of the mothers showed rabies symptoms between 14 to 20 days.

TABLE I. Response of Lactating Hamsters Injected in Flank with 0.1 cc Street Virus Rabies Strain, V308. Suckling removed after 6 exposures. 9 groups.

Appearance of symptoms (days)	Negri bodies
14	+
16	+
16	•
16	+
17	•
20	•
20	•

TABLE II. Response of Suckling Hamster after Weaning from Lactating Parent (Infected with Rabies Street Virus V308).

Group No.	No. of suckling	Onset of rabies (days)	Negri bodies (sucklings)	Mouse inoculation	Negri bodies
1	5	8	+	+	+
		8	+	+	+
		9	+	+	+
		9	+	+	+
		9	+	+	+
2	5	9	+	+	+
		9	+	+	+
		9	+	+	+
		9	+	+	+
3	5	8	+	+	+
		8	+	+	+
		8	+	+	+
		10	+	+	+
		12	+	+	+
4	4	—	—	—	—
5	4	22	+	+	+
6 to 9			Negative		
10[a]	6		(uninoculated controls)		

[a] Showed no clinical symptoms.

31% of the suckling hamsters developed rabies symptoms between 8 to 22 days. Confirmation of rabies was made on all positive animals by Negri body examination. The possibility exists that rabies virus was present in the saliva of the mothers before the suckling animals were weaned, and consequently infection could have occurred by some route other than the milk. This could occur even without biting. Direct infection of mucous surfaces as the result of licking is possible, although this seems remote due to the high percentage of suckling hamsters showing rabies symptoms.

1. Negri, A., *Z. f. Hyg. u. Infektionskr.*, 1903, v45, 507.

2. Reagan, R. L., Day, W. C., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 654.

3. Sellers, V. F., *Am. J. Pub. Health*, 1929, v17, 1080

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5-Hydroxytryptamine in Mast Cells.* (22016)

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Two important physiologically potent tissue constituents have been associated with mast cells: heparin by Holmgren and Wilander(1) and histamine by Riley and West(2). In the present communication, we shall provide evidence that 5-hydroxytryptamine is present in the mast cells of at least one species, the rat.

Mast cells were prepared from the peritoneal cavity of rats by a modification of the method of Padawar and Gordon(3). The cells of the final concentrate appeared well preserved; they maintained their characteristic staining with toluidine blue. There was evidence of some shedding of peripheral granules. Total and differential counts were performed using the solution designed by Moore and James(4) for blood basophiles. Mast cells constituted from 87 to 92%, usually 90 to 92%, of the total population in the final preparations. The remaining cells were mesothelial, mononuclear cells and a few polymorphonuclear leukocytes. Estimates of the volume of cells were made using a red blood cell diluting pipette of about 1 ml capacity centrifuged at 1000 x g for 1.5 hours to constant volume. From 10 rats, 5 to 10 mm^3 of cells were obtained. The mean volume per cell was estimated in 4 determinations to be $1.26 \pm 0.13 \times 10^{-6}$ mm^3.

5-hydroxytryptamine was identified and estimated using the rat colon as described by Dalgleish, Toh, and Work(5). The volume of the bath was 16 ml. For extraction of 5-hydroxytryptamine from the cells the final concentrate in sucrose, containing 1 to 1.4 mm^3 of cells per ml, was diluted 1:20 with distilled water and frozen and thawed 3 times. Although this does not grossly fragment the cells, it does release their histamine and 5-hydroxytryptamine.

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Identification of 5-hydroxytryptamine in the frozen and thawed preparation was made as follows: 1) The material contracted the rat colon in a manner identical with synthetic 5-hydroxytryptamine(6). The capacity of the material in the extract to contract the colon strip was abolished by repeated application of relatively large amounts, 1 to 2 μg of 5-hydroxytryptamine to the strip, as with 5-hydroxytryptamine itself(6). Lysergic acid diethylamide, 10 μg in the 16 ml bath for 10 minutes, abolished the reaction to both extracted material and synthetic serotonin. Dibenamine hydrochloride, 100 μg in the bath, likewise abolished the effect of mast cell extract and reference standard(7). Final identification of the material was made by extraction and chromatography: 6.4 mm^3 of a preparation containing 92% mast cells was extracted 3 times with 95% acetone(6). The acetone was evaporated partly to dryness *in vacuo* at 32°C, applied to Whatman No. 1 filter paper and chromatogrammed using butanol-acetic acid-water (4:1:5)(5). The Rf value for the unknown was 0.44 and of synthetic standard was 0.46. Formaldehyde-bichromate spray and ultraviolet illumination were used for identification(8). The characteristic golden yellow fluorescence was observed.

The concentration of 5-hydroxytryptamine (as free base) was estimated by colon-assay to be 0.63 $\mu g/mm^3$ of cells. From the chromatogram it was estimated to be about 0.7 $\mu g/mm^3$. Evidence that the material is not present in the residual contaminating cells in the mast cell concentrate is as follows: In concentrating the exudate, the 5-hydroxytryptamine activity increases with increasing purity of mast cells. The skin and subcutaneous tissue of the rat contain material with identical activity on the rat colon strip and chromatographic mobility with the butanol-acetic acid-water solvent. The activity of this material

parallels the mast cell content in a manner similar to that previously shown for histamine(9).

The concentration of 5-hydroxytryptamine in the mast cells of the subcutaneous areolar tissue of the rat was computed from estimates of 5-hydroxytryptamine on homogenates of areolar tissue and measurements by the method of Chalkley(10) of the relative mast cell volume. By this means, a value of 0.72 μg 5-hydroxytryptamine per mm^3 of subcutaneous mast cells was obtained.

The evidence given demonstrates the presence of 5-hydroxytryptamine in the mast cells of the rat. We have found that this material has physiological activity when released by those agents which produce mast cell damage(11).

The presence and quantity of histamine and heparin in the isolated concentrated mast cells has been demonstrated using similar technics (12). The amount of histamine was estimated to be 10 $\mu g/mm^3$ and of heparin 30 $\mu g/mm^3$.

Definitive evidence of the presence of 5-hydroxytryptamine in mast cells along with histamine and heparin is important for several reasons: it emphasizes the significance of these cells as participants in the reaction to injury; it provides further evidence for a relationship between mast cells and blood platelets(13); finally, a connection is established between mast cells and the cells of the enterochromaffin system and with certain, as yet unidentified cells in the central nervous system since both of these, as well as the blood platelets, are now known to contain 5-hydroxytryptamine(14,6,15).

Summary. 5-Hydroxytryptamine as well as histamine and heparin was demonstrated to be present in purified mast cells from the peritoneal washings of rats. The material was identified by bioassay and chromatography. The concentration of 5-hydroxytryptamine found in isolated mast cells agreed with that found by indirect means in the mast cells of the subcutaneous areolar tissue of rats. Implications of this finding are briefly discussed.

1. Holmgren, H., and Wilander, O., *T. Mikroskop.-Anat. Forsch.*, 1937, v42, 242.

2. Riley, J. F., and West, G. B., *J. Physiol.*, 1953, v120, 528.

3. Padawar, J., and Gordon, A. S., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 29.

4. Moore, J. E., III, and James, G. W., III, *ibid.*, 1953, v82, 601.

5. Dalgliesh, C. E., Toh, C. C., and Work, T. S., *J. Physiol.*, 1953, v120, 298.

6. Amin, A. H., Crawford, T. B. B., and Gaddum, J. H., *ibid.*, 1953, v126, 596.

7. Gaddum, J. H., and Hameed, K. A., *B. J. Pharmacol.*, 1954, v9, 24.

8. Shepherd, D. M., West, G. B., and Erspamer, V., *Nature*, 1953, v172, 357.

9. Benditt, E. P., Bader, S., and Lam, K. B., *Arch. Path.*, 1955, v60, 104.

10. Chalkley, H. W., *J. Nat. Canc. Inst.* (U.S.) 1943, v4, 47.

11. Benditt, E. P., and Rowley, D. A., *Science*, in press.

12. Benditt, E. P., Lagunoff, D., and Wong, R., to be published.

13. Benditt, E. P., and Mitziga, D. W., *Proc. Chicago Inst. Med.*, 1955, v20, 308.

14. Tucker, M. B., and Rapport, M. M., *Fed. Proc.*, 1954, v13, 170.

15. Erspamer, V., *Rendicont Scientifici Farmitalia*, 1954, v1, 5.

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Species Differences in Serum Glycoproteins of Man, Dog and Rabbit, by Paper Electrophoresis.* (22017)

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Paper electrophoresis provides a simple technic for the identification of serum proteins and stainable components bound to serum proteins. By application of the periodic acid-Schiff method for histochemical identification of various carbohydrate substances, Köiw and Gronwall first adapted this technic for the study of serum glycoproteins(1). The purpose of the present study was to establish a quantitative pattern of these components and their distribution in relation to serum proteins in normal human serum and in plasma from dogs and rabbits.

Procedure. Paper electrophoresis was performed according to a method described elsewhere(2,3). Filter paper strips were run in parallel for subsequent determination of proteins and glycoproteins. For the latter, .040 cc of human serum and rabbit plasma were employed, and .060 cc of dog plasma. In certain instances a third strip was added for determination of lipoproteins (Oil-red-0 stain). The staining procedure employed for glycoproteins differed from that of Köiw and Gronwall in several steps. In preparation of the fuchsin sulfite (Schiff's reagent) 15 to 20 ml of concentrated hydrochloric acid rather than dilute acid were added as a final step. The paper strips were immersed in this solution under constant shaking for time periods varying usually between 10-15 minutes. The staining was continued until clear visibility of the purple bands was obtained without coloring of the background. The densitometric evaluation of the strips was performed immediately afterwards because of their tendency to darken after drying(4). The original color of the strips can be restored by brief immersion in the "sulfite rinse." Individual determinations were performed on serum from 9 normal human beings, six men and three women, ages 15 to 55 years, and clinically free of disease. Heparinized plasma from 6 normal male mongrel dogs and 8 normal male chinchilla rabbits were included in the study. The strips of human serum or animal plasma stained for glycoprotein (glycidograms) revealed 3 main components migrating with the mobility of alpha-1, alpha-2 and beta globulins. These appeared as distinct bands on the paper strips. The densitometric curves were readily separated into these 3 components. Gamma globulin was in our experience devoid of carbohydrate-staining material. Only faint amounts of material stained in the region of albumin; this fraction was therefore not included in evaluating the normal distribution pattern of the glycoproteins. Around the line of application of the serum a small amount of stainable material was usually visible which probably represents stainable substances adsorbed at the point of origin which showed no migration in the electrical field (0-fraction). Accordingly, the patterns were standardized only for the abovementioned main components; alpha-1, alpha-2 and beta glycoproteins. Rough correlation was also observed in man between the total area of glycoprotein stained, measured by the planimeter, and the levels of total serum polysaccharide or hexosamine determined chemically.† This was noted despite the observation that different glycols may vary in their affinity for periodic acid(5,6). Even after filter paper strips had darkened on standing, densitometric evaluation after reimmersion in the "sulfite rinse" yielded curves and planimetric values identical to those obtained on initial examination. With strict adherence to this principle and with careful technic the method was found to be reproducible.

Results. In normal human serum the alpha-2 fraction was the largest (approximately

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† We wish to thank Dr. Ezra Greenspan and Dr. Arthur Ludwig and their technical staff for these determinations.

TABLE I. Glycoprotein Distribution in Various Species.

Species	% of total stainable carbohydrate		
	Alpha-1	Alpha-2	Beta
Man	32.5	39.8	27.6
	24.6	41.1	34.2
	26.8	40.6	32.4
	33.9	39.7	26.4
	23.2	47.4	29.4
	31.7	35.0	33.3
	29.0	42.0	29.0
	31.4	42.9	25.7
	26.0	46.0	28.0
Mean	28.8 ± 3.8	41.6 ± 3.6	29.6 ± 3.1*
Dog	23.5	54.5	22.0
	27.0	52.5	20.5
	20.0	62.5	17.0
	21.0	52.5	26.5
	19.0	63.0	18.0
	29.0	51.5	19.5
Mean	23.2 ± 4.0	56.1 ± 5.3	20.6 ± 3.4*
Rabbit	92.5		7.5
	83.0		17.0
	85.0		15.0
	86.5		13.5
	90.0		10.0
	80.0		20.0
	82.0		18.0
	89.0		11.0
Mean	86.0 ± 4.3		14.0 ± 4.3

* Statistical significance of differences of mean values for beta-glycoprotein: Man vs. dog, $p = .002$; man vs. rabbit, $p = <.0001$.

40%), while alpha-1 and beta were approximately equal, 30% each (Table I). In dogs, three glycoprotein bands were again identified; alpha-2 glycoprotein migrated somewhat more rapidly and was thereby closer in position to alpha-1 glycoprotein than in human serum (Fig. 1). The total quantity of stainable carbohydrate was less, necessitating the application of more plasma for a visible pattern (see procedure). Alpha-2 glycoprotein again was the main component, accounting, however, for even a greater percentage of the total stainable carbohydrate than in man (approximately 56%); alpha-1 and beta glycoprotein were present in about equal amounts but in lower proportion than in human serum (Table I). In rabbits, separation of alpha-1 and alpha-2 glycoprotein was not possible in many instances and, therefore, a single alpha-glycoprotein component was measured planimetrically. It was the major plasma glycoprotein component (approximately 86%) (Table I, Fig. 1). Beta glycoprotein was in this species considerably lower than in the other two.

No alteration of the normal plasma glycoprotein pattern of the rabbit was noted in 6 animals treated with corticosteroids [prednisone (Δ_1-Dehydrocortisone), cortisone, hydrocortisone] for 3-5 weeks nor in 3 rabbits in which hypercholesteremia had been induced.‡ In man, in several instances of multiple myeloma and liver disease a gamma glycoprotein band appeared in addition to the usual components and could be clearly separated from the 0-fraction.

Discussion. In this study a quantitative pattern of glycoprotein distribution in the serum (plasma) of man, rabbit and dog has been established by paper electrophoresis. To our knowledge, no previous report of such a study is available. Other investigators(1, 4, 7-9) have reported qualitative patterns of serum glycoproteins in normal man and in various disease states(1,4). Some of these patterns differed from those obtained in this study in that a gamma-glycoprotein was described in normal serum. The carbohydrate-staining fraction with slowest mobility (or perhaps no mobility) in our experience corresponded to that material adsorbed at the point of application of the serum (0-fraction). It did not move with the gamma globulin which under our experimental conditions migrated "backwards," *i.e.*, toward the cathode. In certain pathological states, however, a "true" gamma glycoprotein has been observed by others(1,4,7-9) and by ourselves. In all reports except that of Köiw and Gronwall, alpha-2 glycoprotein was the major component of the serum; in their graph, the largest glycoprotein peak was alpha-1.

The species examined showed distinctive patterns of glycoprotein distribution in a similar fashion to the species-specific protein and lipoprotein patterns described previously(2,3, 10,11). The glycoprotein distribution in man differed in a statistically significant manner from that obtained in dog and rabbit, with most marked divergence between man and rabbit. Previous examination of lipoproteins revealed a similar distribution pattern in man

‡ We are obliged to Dr. Chun-I Wang for providing these animals.

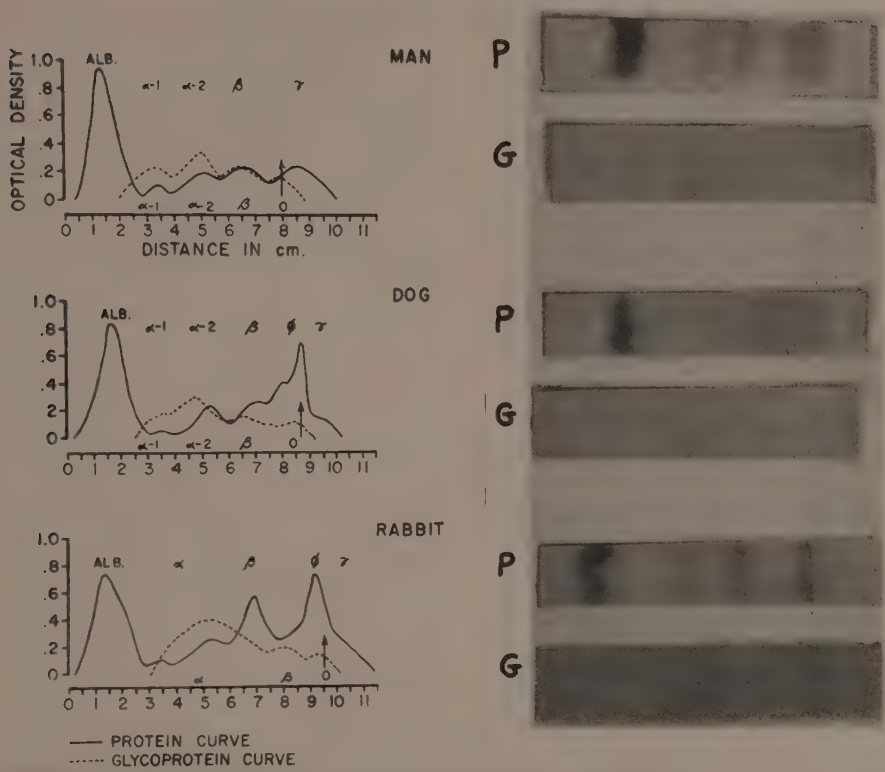

FIG. 1. Protein and glycoprotein electropherograms in man, dog and rabbit. On left are curves obtained by densitometric evaluation, on right are stained paper strips. P = Paper strip stained with amidoschwarz for protein. G = Paper strip stained with PAS for carbohydrate. Arrow in graphs and ruled line on paper strip represent the point of application of the serum (plasma). For details, see text.

and rabbit with regard to alpha and beta lipoprotein whereas in the dog the pattern was reversed. Thus, while species variations in these plasma components exist, they are not of parallel magnitude. Establishment of such normal patterns permits more detailed characterization of alterations in pathological states.

The technic of paper electrophoresis and subsequent periodic acid-Schiff stain did not yield values for distribution of serum glycoproteins comparable to those obtained by chemical analysis of plasma protein fractions (Cohn). The two procedures differed markedly and some discrepancy in results was not surprising; *e.g.*, alpha-1 glycoprotein appeared to be lower with the Cohn-fractionation technic. In addition, the analytical results of Cohn fractions differed with the author(12,13).

Summary. (1) By means of paper electrophoresis and subsequent carbohydrate staining by the periodic acid-Schiff method, quantitative evaluation of species glycoprotein patterns were established for man, dog and rabbit. (2) This procedure provides a simple, reproducible method for the quantitative study of serum glycoproteins which may be correlated with simultaneous determination of proteins and lipoproteins. (3) Carbohydrate was bound to alpha-1, alpha-2 and beta-globulins, with the major portion corresponding to the alpha globulins. Gamma glycoprotein was absent in normal serum in man. (4) No alterations were observed in rabbits after administration of adrenal cortical ster-

oids nor in experimentally-induced hypercholesteremia.

1. Köiw, E., and Gronwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 245.

2. Bossak, E. T., Wang, C. I., and Adlersberg, D., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 637.

3. Adlersberg, D., Bossak, E. T., Sher, I. H., and Sobotka, H., *Clin. Chemistry*, 1955, v1, 18.

4. Rice, W. G., *J. Lab. and Clin. Med.*, 1954, v44, 544.

5. Jeanloz, K., *Science*, 1950, v111, 289.

6. Gomori, G., *Am. J. Clin. Path.*, 1952, v22, 277.

7. Osserman, E. F., and Lawlor, D. P., *Science*, 1954, v120, 715.

8. Sachs, B. A., Cady, P., and Ross, G., *Am. J. Med.*, 1954, v17, 663.

9. Kuhns, W. J., *J. Exp. Med.*, 1954, v100, 485.

10. Bossak, E. T., Wang, C. I., and Adlersberg, D., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 634.

11. Bossak, E. T., Wang, C. I., and Adlersberg, D., *J. Clin. Endo. and Metab.*, in press.

12. Seibert, F. B., Pfaff, M. L., and Seibert, M. V., *Arch. Biochem.*, 1948, v18, 278.

13. Lever, W. F., and Hurley, N. A., *The Plasma Glycoproteins and Lipoproteins* in *Blood Cells, and Plasma Proteins*, edited by James L. Tullis.

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ERRATUM

In Vol. 88, 1955, article on rapid virulence test in diphtheria, page 369, line 8, 0.0045% should read 0.033%.

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SECTION MEETINGS

CLEVELAND, O.	
Western Reserve University	October 17, 1955
PACIFIC COAST	
University of California	October 12, 1955
SOUTHERN CALIFORNIA	
University of Southern California	October 20, 1955

Influence of Yolk on Mitotic Rate in Untreated and X-Rayed Grasshopper Neuroblasts *in vitro*.* (22018)

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(Introduced by Alexander Hollaender.)
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The 14-day-old embryo of the grasshopper *Chortophaga viridifasciata* (De Geer) has been extensively used by Carlson, Gaulden, and coworkers for *in vitro* studies of its large, rapidly dividing neuroblasts. In the course of some experiments requiring rapid preparation of hanging-drop cultures of irradiated embryos, the small amount of grasshopper egg yolk usually added to the artificial medium (1) in the cultures was omitted to speed operations. The recovery of the neuroblasts in these cultures from radiation-induced mitotic inhibition was greatly retarded. The present report describes a detailed examination of the influence of yolk on mitotic rate in untreated neuroblasts and on recovery from mitotic inhibition in X-rayed neuroblasts. A positive influence was found in each case.

Materials and methods. In all experiments embryos were dissected and made into hanging-drop preparations for *in vitro* observations as described by Gaulden, Nix, and Moshman (2), with two exceptions. Following separation from their membranes and removal of their appendages (hereafter referred to as "dissection"), the embryos were left surrounded by yolk in a dish of artificial culture medium until they were mounted in hanging-drop cultures. Varying amounts of yolk, or no yolk, were added to the cultures. For study of the influence of yolk on mitotic rate of untreated embryos, 4 different amounts were tested, namely, approximately 0, ⅛, ¼, and ½ the amount present in an egg. Amounts of yolk greater than ½ were not used because they increased greatly the osmotic pressure of the culture drop resulting in a change in mitotic rate(3). With the amounts of yolk used in the present experiments, no changes in osmotic pressure—as determined by the visible appearance of cells and chromatin—were observed except possibly during the later counting periods in embryos mounted with ½ yolk. A given amount of yolk can be obtained by placing a 14-day-old egg (Fig. 1) in a dish of culture medium,

* Work performed for the Atomic Energy Commission under Contract No. W-7405-eng-26.

cutting off the posterior tip of the egg, and gently pressing the embryo and yolk out of the chorion. If this operation is performed carefully, the yolk comes out in the form of a cylinder with the embryo lying on top at one end. The embryo and its membranes can be easily discarded without disturbing the yolk. The cylinder of yolk can then be cut into portions of desired size. The anterior ⅛, ¼, or ½ of the yolk cylinder was used because its consistency was more uniform than that of the posterior portion. Currents in the medium around the yolk are kept at a minimum during these operations in order to prevent dispersal of the serosal fluid which surrounds the yolk. Twelve minutes after dissection each embryo was mounted on a cover glass, ventral surface down, in a drop of culture medium to which was added a given amount of yolk or no yolk. The total amount of culture material in the hanging-drop was equal to 5-10 μl of fluid. The cover glass was inverted and sealed over a depression slide with thick mineral oil. The volume of the depressions used varied from 0.0531 to 0.0594 ml. Twenty-one minutes after the embryos had been dissected the hanging-drop preparations were placed on the stage of a microscope, the lower portion of which was enclosed in an incubator maintained at 38° ± 1°C. Observations on the neuroblasts, which lie just beneath the cover glass in these preparations and which are in all stages of mitosis, were begun 1 minute later with the oil immersion lens and bright-field illumination. Light from the microscope lamp was passed through a 5% solution of copper sulfate to remove heat. For study of the influence of yolk on recovery of neuroblasts from X-ray-induced mitotic injury, embryos were exposed *in vivo* to 64 r of X rays at room temperature (25° ± 2°C). A General Electric Maxitron 250-kvp unit was used at 125 kvp and 15 ma with 3.5 mm Al filtration and 54.5 cm target distance. The dose rate was 32 r/minute. Embryos were dissected 18 minutes after irradiation and mounted 2 minutes later. In preparation of the hanging-drop cultures, the X-rayed embryos were mounted, half with ¼ yolk and half without yolk. Untreated control embryos were similarly mounted. Twenty-one minutes after irradiation, the hanging-drop cultures of both treated and control embryos were placed in the incubator at 38° ± 1°C and observations begun 1 minute later.

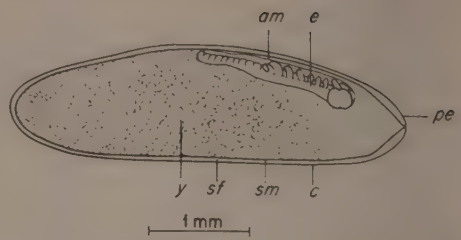

FIG. 1. *Camera lucida* sketch of 14-day-old egg of grasshopper, *Chortophaga viridifasciata*. *am*, amnionic membrane; *c*, chorion and closely adhering cuticle layers; *e*, embryo; *pe*, posterior end of egg; *sf*, serosal fluid; *sm*, serosal membrane; *y*, yolk.

The method for determining mitotic rate is as follows. Approximately 140 neuroblasts per embryo (all neuroblasts in the 3 thoracic segments) are examined at 16 22-minute intervals and the number of neuroblasts in mid-mitosis (prometaphase, metaphase, and anaphase) are recorded. Such counts provide a valid determination of the number of cells going through mitosis in a given period of time because the duration of mid-mitosis at 38°C is 22 minutes under the conditions of the present experiments. The short interval between observation periods limits the number of embryos that can be examined at one time. Thus the data for each experimental condition represent a compilation of data obtained from several sets of embryos studied on several successive days. In the experiments to test the influence of yolk on untreated mitosis, 2 embryos with each of the 3 amounts of yolk and 2 with no yolk were prepared for study at a time. In the X-ray experiments, 4 X-rayed and 4 untreated embryos, 2 with and 2 without yolk in each case, were studied at a time.

Results. Results of the experiments designed to determine the influence of yolk on mitotic rate of untreated neuroblasts are given in Fig. 2 as the average number of mid-mitotic cells per embryo plotted against time after dissection. An analysis of variance test† of

† The authors are indebted to Dr. A. W. Kimball and Mr. George Atta for the statistical analyses of the data.

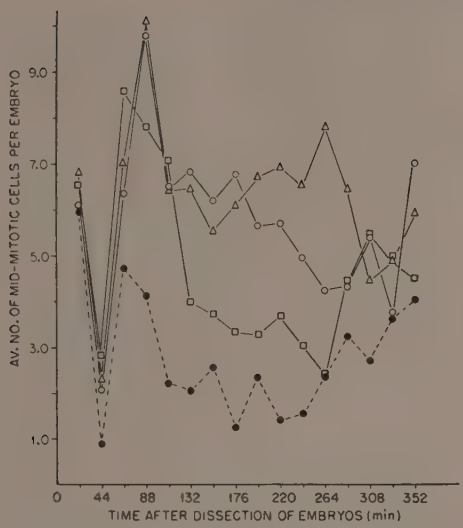

FIG. 2. Effects of varying amounts of yolk from a grasshopper egg on mitotic rate of untreated neuroblasts. ●, no yolk (8 embryos); □, ⅛ yolk (9 embryos); △, ¼ yolk (8 embryos); ○, ½ yolk (7 embryos).

the raw data revealed that the shapes of the curves are essentially the same but that their over-all heights are different at the 0.1% level of significance. The number of mid-mitotic cells in the no-yolk cultures as compared with the yolk cultures at the 44- and 66-minute counts (Fig. 2) was significantly lower only at the 5-10% level of probability. This cannot be considered a statistically significant difference, but it does indicate an early trend toward low mitotic activity in the no-yolk cultures that reaches significant proportions by the 88-minute count. In other words, the decrease in number of cells that can complete mitosis in the absence of nutritive yolk becomes evident soon after the cells are separated from the yolk.

The drop in the frequency of mid-mitotic cells at the 44-minute count followed by a rise at the 66- and/or 88-minute counts occurred in all 4 types of culture preparations. Exploratory experiments revealed that this fall and rise of activity results from a brief inhibition of mitotic progress of cells which enter or are in middle and late prophase when cultures are prepared. This inhibition appears to be caused partly by temperature shock that results when cultures are transferred from room temperature to 38°C and partly by the slight adjustments in cellular osmotic pressure which occur immediately following the change from *in vivo* to *in vitro* conditions.

In Fig. 3 is shown the relation between amount of yolk in the culture preparation to the mean number of cells completing mitosis in 352 minutes. It can be seen that the frequency of cells going through mitosis increased as the quantity of yolk increased up to ¼‡. The mean mitotic frequency in cultures containing ½ yolk was a little lower than that in the ¼-yolk cultures. This is probably caused by adverse effects of the slightly higher osmotic pressure that develops in the medium of the ½-yolk cultures about 3.5 hours after they are prepared. The cause of this increased osmotic pressure has not yet been determined. In cultures made up with amounts of yolk greater than ½, the increased osmotic pressure becomes manifest very soon after their preparation. For this reason larger amounts of yolk were not used in the present study.

Results of the experiments designed to de-

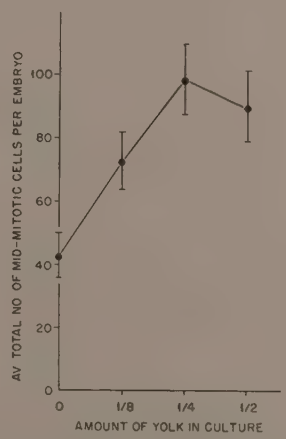

FIG. 3. Total number of mid-mitotic neuroblasts per embryo as function of amount of yolk in culture drop. The interval about each point represents the region within the 95% confidence limits.

‡ It should be noted that an amount of yolk approximately equal to ¼ that in an egg has been routinely used in preparing hanging-drop cultures in all previous work on grasshopper neuroblasts.

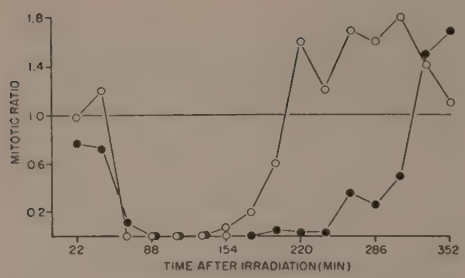

FIG. 4. Effects of yolk on recovery of neuroblasts from X-ray (64 r) induced injury. ○, mitotic ratio of X-rayed cells : control cells mounted with ¼ yolk; ●, mitotic ratio of X-rayed cells : control cells mounted without yolk. Data obtained from 8 embryos for each experimental condition.

termine the influence of yolk on recovery of neuroblasts from X-ray-induced mitotic inhibition are given as mitotic ratios in Fig. 4. It can be readily seen that the duration of the inhibition period is shorter and the beginning and completion of recovery is considerably faster in the neuroblasts of embryos mounted in yolk than in those of embryos mounted without yolk. These differences are statistically significant at the 1% level as shown by an analysis of variance of the raw data. In other words, yolk greatly enhances the recovery of neuroblasts from X-ray-induced mitotic injury.

Discussion. Untreated neuroblast. The decrease in mitotic rate which is evident soon after neuroblasts are separated from yolk suggests that the neuroblast does not go through a given mitosis entirely on energy previously stored within the cell. The frequency of mid-mitotic cells in the no-yolk cultures reaches a statistically significant low level at the time (88-minute count) that the frequency in the yolk cultures is at its maximum (Fig. 2). This high frequency in yolk cultures is the result of recovery of middle and late prophase cells from a temporary inhibition induced by change from *in vivo* to *in vitro* conditions. It would appear, therefore, that the absence of yolk considerably affects the ability of middle and late prophase cells to complete mitosis.

Under optimum *in vitro* growth conditions, cells which were in early prophase and earlier stages or even in the previous mitosis would be expected to reach mid-mitosis at the 88-minute and successive counts.§ Since the frequencies of mid-mitotic cells in the no-yolk cultures are very low at these counts, it appears that cells in early prophase and earlier stages are unable to complete mitosis in the absence of yolk.

Cells in stages from very late prophase through anaphase either require very little energy for their processes or they operate on energy stored within the cell during earlier phases. The second explanation seems the more likely, especially in view of the considerable amount of activity, such as chromosome movement and cleavage furrow formation, that occurs during these stages.

Several investigators are of the opinion that a cell is capable of completing mitosis without acquiring additional energy after the process has begun. Bullough(4,5) has data on mitosis in mouse epidermis which he interprets as evidence that these cells need considerable amounts of energy to initiate mitosis but very little energy to complete it. Swann(6,7) has proposed, on basis of his work with developing sea urchin eggs and that of Jacoby, *et al.* (8) on chick fibroblasts, that the energy necessary for a given mitosis is supplied by the "siphoning" of an energy "reservoir" which is stored during the previous mitosis. In other words, conditions which adversely affect the energy supply of the cell would not be detected until the subsequent division. The results herein reported suggest that in the grasshopper neuroblast at least the first half of prophase does not operate solely on energy stored within the cell but is dependent on an outside source of energy for continuance of mitosis.

That the energy for division is derived from glycolysis seems a reasonable conclusion to be drawn from the results of several workers (9-12). Tipton and St. Amand(13) have shown, however, that glucose does not influence respiration of the 14-day-old embryo of the grasshopper *Chortophaga viridifasciata*. This is not surprising in view of the findings

§ These calculations are based on durations of the various mitotic phases as given by Carlson and Hollaender, *J. Cell. and Comp. Physiol.*, 1948, v31, 149.

of Bodine(14) and Hill(15) that fat seems to be the main source of energy for grasshopper embryos of this age.

Fischer(16) has found that the growth of chick myeloblasts and osteoblasts *in vitro* is dependent on several amino acids, cysteine being the most essential. Shaw(17) has identified 17 free amino acids in the 14-day-old egg of *Chortophaga*. However, since his analysis is based on entire contents of the egg, it is difficult to estimate the possible role of these amino acids in mitosis of the neuroblast.

Preliminary experiments in this laboratory indicate that a filtrate‖ of fresh grasshopper egg contents gives the same result as ¼ yolk. The filtrate contains miscroscopic globules of yellow "yolk" material but no other visible components.

Irradiated neuroblast. It is not known whether recovery from radiation-induced mitotic inhibition involves the replacement of some vital substance which was destroyed by radiation or the elimination of some substance which caused the inhibition(18). The pronounced positive influence of yolk on recovery of neuroblasts from radiation damage demonstrates that in this cell some extracellular metabolite or metabolites greatly enhance repair of mitotic damage. This result strikingly parallels that of Stapleton and coworkers(19,20,21) who have found that the addition of beef or yeast extracts, or amino acids to culture medium following irradiation causes considerable increases in the number of bacteria which survive the radiation. The amino acids in the grasshopper egg(17) may, therefore, be important factors in the recovery of the neuroblast from radiation damage.

The shapes of the curves in Fig. 4 suggest that the response of neuroblasts to 64 r of X rays in the absence of yolk differs from the response in presence of yolk only with respect to the duration of the inhibition period. The temporary retardation of cells in very early, early, middle and late prophase is mainly responsible for the inhibition period in the presence of yolk. Whether the absence of yolk affects recovery of cells in all these stages to the same extent or whether it differentially affects recovery of certain ones cannot be determined from the present data.

‖ The extraembryonic contents of 14-day-old grasshopper eggs were mixed in 10 volumes of culture medium and passed through a fine Seitz filter. Culture medium rather than plain water was used because yolk and, in particular, serosal fluid contain much protein, presumably globulin, which readily coagulates in water.

Summary. 1. The frequency of mid-mitotic neuroblasts in grasshopper embryos in hanging-drop cultures increases as the quantity of yolk in the culture is increased up to a quantity equivalent to ¼ that in an egg. The influence of yolk on mitosis in the untreated neuroblast is interpreted as indicating that, in this cell at least, the first half of prophase does not operate solely on energy stored within the cell but is dependent on an outside source of energy for continuance of mitosis. 2. The pronounced positive effect of yolk on recovery of neuroblasts from radiation damage demonstrates that, in this cell, some extracellular metabolite or metabolites greatly enhance repair of mitotic damage.

1. Carlson, J. G., Hollaender, A., and Gaulden, M. E., *Science,* 1947, v105, 187.

2. Gaulden, M. E., Nix, M., and Moshman, J., *J. Cell. and Comp. Physiol.,* 1953, v41, 451.

3. Gaulden, M. E., 1955, in mss.

4. Bullough, W. S., *J. Endocrinol.,* 1950, v6, 350.

5. ———, *Biol. Rev.,* 1952, v27, 133.

6. Swann, M. M., *Quart. J. Microscop. Sci.,* 1953, v94, 369.

7. ———, in *Recent Developments in Cell Physiology,* J. A. Kitching, ed., 1954, Academic Press, p185.

8. Jacoby, F., Trowell, O. A., and Willmer, E. N., *J. Exp. Biol.,* 1937, v14, 255.

9. Bullough, W. S., *J. Exp. Biol.,* 1949, v26, 83.

10. Brown, R., and Rickless, P., *Proc. Roy. Soc. Lond.,* (B), 1949, v136, 110.

11. O'Connor, R. J., *Brit. J. Exp. Path.,* 1950, v31, 390.

12. Lettre, H., *Cancer Research,* 1952, v12, 847.

13. Tipton, S. R., and St. Amand, G. S., *Physiol. Zool.,* 1954, v27, 311.

14. Bodine, J. H., *ibid.,* 1946, v19, 54.

15. Hill, David L., *J. Cell and Comp. Physiol.,* 1945, v25, 205.

16. Fischer, A., *Biochem. J.,* 1948, v43, 491.

17. Shaw, E. I., *Exp. Cell. Research,* 1955, in press.

18. Lea, D. E., *Actions of Radiations on Living*

Cells, 2nd Ed., 1955, Cambridge Univ. Press.
19. Stapleton, G. E., *Ann. N. Y. Acad. Sci.*, 1955, v59, 604.
20. Stapleton, G. E., Billen, D., and Hollaender, A., *J. Cell. and Comp. Physiol.*, 1953, v41, 345.
21. Stapleton, G. E., Sbarra, A. J., and Hollaender, A., *J. Bact.*, 1955, v70, 7.

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Metabolism of Trichloroethylene in the Bovine.* (22019)

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It has been suggested(1) that the aplastic anemia which is observed in the bovine(2) fed certain specimens of trichloroethylene-extracted soybean oil meal is induced by residual trichloroethylene in the toxic meal. Prolonged feeding of relatively large amounts of trichloroethylene however failed to produce harmful effects in calves(3). This suggests that the bovine, like some other species which are relatively resistant to the toxic effects of trichloroethylene-extracted soybean oil meal (4) can metabolize trichloroethylene. In fact, urochloralic acid has recently been isolated from the urine of calves fed trichloroethylene and identified through degradation and synthesis of a derivative as 2′, 2′, 2′-trichloroethyl-β-D-glucopyranosiduronic acid(5). It was thus established that the bovine, like the dog(6), can metabolize trichloroethylene to trichloroethanol and excrete the latter as a conjugate. It remained to be determined however, to what extent this occurs and if trichloroacetic acid, a major metabolite of trichloroethylene in mice(7), rats(8), dogs (9) and man(10) is also produced by the bovine.

Methods. Four female Holstein calves weighing from 100 to 112 lb were placed into individual metabolism cages, fed 5 lb of whole cows' milk twice daily and given water *ad libitum*. Trichloroethylene was administered orally in gelatin capsules twice daily with the milk in the following total daily amounts: Calf A, 3 g for 5 days; calf B, 12 g for 5 days; calves C and D, 12 g for 4 days. The urine was collected in brown bottles under toluene. Separate 24-hour specimens were obtained before, during and after the period of administration of trichloroethylene. Aliquots of the urine were used for determination of trichloroacetic acid, "total trichloro compounds" and trichloroethylene; the latter was also determined in the toluene layer. The analytical methods used have been described recently(11). Briefly they consist in the quantitative application of the Fujiwara alkali-pyridine reaction. In our modification trichloroacetic acid, trichloroacetaldehyde, chloroform and trichloroethanol do not interfere with the determination of trichloroethylene. The latter as well as chloroform can be removed from the urine prior to the determination of trichloroacetic acid or of "total trichloro compounds." Trichloroethanol and urochloralic acid do not interfere with the determination of trichloroacetic acid but all 3 compounds are included in the determination of "total trichloro compounds." The difference between "total trichloro compounds" and trichloroacetic acid (calculated on a molar basis as trichloroethanol) corresponds to free and combined "trichloroethanol" including urochloralic acid. Other products of metabolism of trichloroethylene which no doubt exist have not been definitely recognized. If, after oxidation they would give a positive Fujiwara reaction they would be included as "total trichloro compounds" and would thus contribute

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TABLE I. Trichloroacetic Acid and "Trichloroethanol"* in Urine.

24 hr interval	Calf A (3 g/day) TCA†	Calf A (3 g/day) TCE‡	Calf B (12 g/day) TCA†	Calf B (12 g/day) TCE‡	Calf C (12 g/day) TCA†	Calf C (12 g/day) TCE‡	Calf D (12 g/day) TCA†	Calf D (12 g/day) TCE‡
	mg							
1	11.8	500	16.2	1169	0	793	0	911
2	18.6	596	43.1	1339	7.3	613	14.6	692
3	33.6	848	109	2487	15.8	1453	37.4	2116
4	48.3	1475	78.0	1346	37.2	2350	59.3	2595
5	46.6	576	145	1973	79.3	1651	97.9	1697
§								
6	36.5	75	121	116	20.7	39.5	28.2	53
7	25.0	8.6	73.5	16.4				
8	16.9	0	37.2	5.5				
9	16.6	0	38.1	0				
10	11.7	0	16.8	0				

* Includes urochloralic acid and other compounds which give a positive Fujiwara reaction after oxidation.

† Trichloroacetic acid.

‡ "Trichloroethanol" corrected for trichloroacetic acid excreted.

§ Daily dose of trichloroethylene given in parentheses below letter of each animal; fed on days above horizontal line.

to the fraction which, with this reservation in mind, is referred to as "trichloroethanol."

Results. No ill effects on the calves were observed as a result of the administration of trichloroethylene. No compounds giving a positive Fujiwara test were detected in the urine collected before the administration of trichloroethylene.

Trichloroethylene could not be detected in the aqueous phase of the collected specimens even after extensive aeration and collection of the volatile components in toluene. However, while trichloroethylene was fed, the toluene layer used to preserve the urine contained from 3 to 6 micromoles of trichloroethylene per day which in the case of calves B to D is less than 0.01% of the dose fed.

The presence of trichloroacetaldehyde, an assumed product of metabolism of trichloroethylene(6) and the probable direct precursor of trichloroacetic acid and trichloroethanol could not be detected by a test(11) which is sensitive to at least 15 μg; lack of evidence for the presence of trichloroacetaldehyde is in accord with the observations of Butler with dogs(6).

Table I summarizes the data obtained with determinations of trichloroacetic acid and "trichloroethanol" in the urine. Although trichloroacetic acid was not identified as such in the urine it was detected through its behavior in the Fujiwara reaction(11). During the administration of trichloroethylene, the concentration of trichloroacetic acid in the urine increased gradually; it decreased again gradually after feeding of trichloroethylene had ceased. The molar recovery of trichloroacetic acid in the urine of calves A and B was only 1.4 and 1% respectively of the total amount of trichloroethylene administered and only 5.6 and 7.2% respectively of the "total trichloro compounds" found in the urine. Continued excretion of trichloroacetic acid after exposure to trichloroethylene has also been observed in humans and in dogs(6). However, in those species the proportion of trichloroethylene metabolized to trichloroacetic acid appears to be much higher than in the bovine. Butler estimated that in dogs (6) trichloroacetic acid was excreted to the extent of about ⅓ to ½ of the amount of trichloroethanol. In the urine of rats(8,12) however, exposed to 80 mg of trichloroethylene per liter of air, the concentration of trichloroacetic acid was only 1.2 mg per liter. Ahlmark and Forssman(8) found that man excreted from 6 to 18% of inhaled trichloroethylene as trichloroacetic acid in the urine. The highest concentration of trichloroacetic acid (50 mg per liter) was found in calf B on the fifth day. In man, Grandjean *et al.* (13) have found that a mean of 13% of the

trichloroethylene inhaled during a working day was excreted as trichloroacetic acid; the concentration of trichloroacetic acid in the urine of their subjects ranged from 8 to 440 mg per liter with a mean of 84.7 mg.

"Trichloroethanol" was excreted by the calves rapidly and extensively after administration of trichloroethylene. The largest amount, 2.6 g was found in the urine of calf D on the fourth day after feeding trichloroethylene; the concentration was 940 mg per liter in this specimen of urine. When feeding of trichloroethylene was discontinued "trichloroethanol" disappeared from the urine within 2 to 3 days. Feeding of trichloroethylene at the higher level increased the excretion of "trichloroethanol" but not proportionately. The recovery of "total trichloro compounds" in the urine during the whole period was only 25.4, 13.3, 13.5 and 15.2% respectively for calves A through D, calculated on a molar basis. Our analytical procedure did not permit differentiation between free and combined trichloroethanol. Butler (6) has reported that dogs excreted from 42 to 66% of trichloroethanol which was injected intramuscularly, mostly in conjugated form.

Discussion. We are not aware of experiments in which the metabolism of trichloroethylene was studied after oral administration; for this reason and because the bovine has not previously been used for studies of the metabolism of trichloroethylene comparisons with other species are somewhat tenuous. However, these experiments show that some of the products of metabolism of trichloroethylene in the bovine are similar to those observed in other species. While it is evident that the calf can "detoxify" trichloroethylene to a considerable extent and excrete urochloralic acid as well as trichloroacetic acid a major fraction of the trichloroethylene administered remained unaccounted for. It is possible that this portion may have been in part exhaled or excreted with the feces, or metabolized to monochloroacetic acid(14) or to inorganic chloride(15) and thus would have escaped detection by the Fujiwara test.

Summary. Following the oral administration of 3 to 12 g of trichloroethylene to calves weighing about 100 lb, about 1% of this compound was excreted in the urine as trichloroacetic acid and from 13 to 25% as trichloroethanol (free and combined). Only traces of trichloroethylene were excreted in the urine. No trichloroacetaldehyde could be demonstrated in the urine with the method used. Some of the metabolic products of trichloroethylene in the bovine are the same as those produced by man and dogs.

1. Menzani, C., *Atti Soc. Ital. Vet.*, 1949, v3, 493.

2. Pritchard, W. R., Rehfeld, C. E., and Sautter, J. H., *J. Am. Vet. Med. Assn.*, 1952, v121, 1.

3. Pritchard, W. R., Mattson, W. E., Sautter, J. H., and Schultze, M. O., *Am. J. Vet. Res.*, in press.

4. Perman, V., Rehfeld, C. E., Sautter, J. H., and Schultze, M. O., *J. Agric. Food Chem.* in press.

5. Schultze, M. O., and Seto, T. A., Abst. 128th Meeting Am. Chem. Soc., Minneapolis, Sept., 1955, 49c., *J. Am. Chem. Soc.*, in press.

6. Butler, T. C., *J. Pharmacol. Exp. Therap.*, 1949, v97, 84.

7. Friberg, L., Kylin, B., and Nystrom, A., *Acta. Pharmacol. Toxicol.*, 1953, v9, 303.

8. Ahlmark, A., and Forssmann, S., *Act. Physiol. Scand.*, 1951, v22, 326.

9. Barrett, H. M., and Johnston, J. H., *J. Biol. Chem.*, 1935, v127, 765.

10. Powell, J. F., *Brit. J. Indust. Med.*, 1945, v2, 142.

11. Seto, T. A., and Schultze, M. O., *Anal. Chem.* in press.

12. Forssmann, S., and Holmquist, C. E., *Acta Pharmacol. Toxicol.*, 1953, v9, 235.

13. Grandjean, E., Münchinger, R., Turrian, V., Haas, P. A., Knoepfel, H. K., and Rosenmund, H., *Brit. J. Ind. Med.*, 1955, v12, 131.

14. Vlachova, D., *Pracovni Lekarstvi*, 1955, v7, 98; *Chem. Abstr.*, 1955, v49, 9083.

15. Bray, G. H., Thorpe, W. V., and Vallance, D. K., *Biochem. J.*, 1952, v51, 193.

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Eosinophiles in Passive Anaphylaxis.* (22020)

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It has long been recognized that blood and tissue eosinophilia is a common accompaniment of antigen-antibody activity(1,2,3). The mechanism responsible for the eosinophilia, however, has not been clear. To determine whether a state of active antibody production is a prerequisite for its development the following experiments on passive anaphylaxis were undertaken.

Materials and Methods. Three times crystallized egg albumin (Ea) was used as antigen. The antiserum was from a rabbit ($R194_{4+5}$, ref. 4), which had been injected with a total dose of 40 mg of antigen over a 2 months' period. This antiserum had a precipitable anti-Ea N content at the time of use of 308 μg per ml. Animals for sensitization and for controls were 250 to 325 g guinea pigs. Injections were made into leg veins. Sensitization was accomplished with 23 μg antibody N given intravenously 48 hours prior to the shocking dose of antigen (6 μg Ea N). Blood counts were done on peripheral blood, use being made of ear, leg, and foot veins for this purpose. To prevent coagulation of the blood, it was necessary to wipe the cleaned skin surface and puncture needle (#23 hypodermic needle) with a piece of cotton previously dampened with heparin. White cell counts were performed in a Spencer bright-line counting chamber. Bloods for the total leukocyte counts were diluted 20-fold in a white cell pipette with a solution of acetic acid and methylene blue. Eosinophile counts were done on 10-fold diluted blood in a freshly prepared solution of Phloxin in 0.1% propylene glycol. Both sides of the counting chamber were filled in each instance, four large squares on each side being used in enumerating the total white count and the entire ruled area being used in enumerating the absolute eosinophile count.

TABLE I. Absolute Eosinophile Counts after 6 μg Egg Albumin Nitrogen Intravenously in 9 Sensitized and 7 Non-Sensitized Guinea Pigs.

	Time in hr							
	0	¼–½	1	2	4	6	12	24
				Sensitized with 23 μg anti Ea N				
	201		195	294	165	243		
	33	51	198	165		357		
	66	171		120	141	207		
	30		81	360	240	204		204
	33		6	255	90	123		162
	33	15	99	381	174	147	78	
	12	87	315	135	105	126		
	27	21	117	99	33	21		
	24	57	111	174	288	21		
Mean ± S.E.	51 ± 19	67 ± 24	140 ± 34	220 ± 35	155 ± 29	161 ± 36		
				Non-sensitized controls				
	18	3	24	18	36	21		9
	36	30	27	36	45	36		9
	30	21		30	36	39		30
	30	6	174	36	42	72	27	12
	45	15	66	15	21	9	18	9
	30	15	15	27	18	21		
	3	9	18	24	12	24		
Mean ± S.E.	27 ± 5.1	14 ± 3.4	54 ± 25	29 ± 3.0	30 ± 4.9	32 ± 7.7		
P	>.2	.04	.08	<.01	<.01	<.01		

* Supported by a grant from the A. D. Williams Fund of the Medical College of Virginia.

† A. D. Williams Postdoctoral Fellow.

TABLE II. Total Leukocyte Counts after 6 μg Egg Albumin Nitrogen Intravenously in 9 Sensitized and 7 Non-Sensitized Guinea Pigs.

	Time in hr							
	0	½	1	2	4	6	12	24
	Animals sensitized with 23 μg anti Ea N							
	9400		9150	13400	9400	9020		
	2150	8400	10800	11100		7600		
		18850		9050	9050	9400		
	6100			14400	6200	7900		8800
	9800			9750	5350	10400		11900
	9750	12200	15750	7950	3100	9650	9250	
	9950	11300	11050	10950	8400	6600		
	14400	9550	8300	12400	4650	5050		
	7150	8500	6850	9800	11100	11300		
Avg	8600	11500	10300	11000	7160	8550		
	Non-sensitized controls							
	5200	10200	8000	11300	8600	16200		5750
	12900	13100	9500	10100	10000	13700		11400
	4900	4450		8900	7200	9150		6300
	6150	5200	6700	5250	6000	3950	7600	5900
	16200	8850	8100	9750	5900	5150	10250	11750
	22850	11200	16450	22050	21000	21850		
	7550	4600	15450	5600	850	4600		
Avg	10550	8230	10600	10400	8510	10700		

Results. Table I and Fig. 1 present the eosinophile counts and Table II presents the total white cell counts in the animals studied. It is clear that there was a significant rise of the eosinophiles in sensitized animals injected with 6 μg of Ea N. This eosinophilia occurred independently of any corresponding rise in the total number of white cells and was maintained for 24 hr in 2 animals examined at this interval. In spite of some variability in the serial counts in these animals, probably due to technical difficulties, the eosinophile differences observed at 2, 4 and 6 hours were statistically highly significant.

It was of interest to note in several instances in a preliminary part of this study moderate eosinophile responses in normal, non-sensitized animals after large (160 μg) doses of Ea N (Table III). This effect was not seen in normal animals at the smaller doses of Ea used later in the anaphylaxis experiments (Table I).

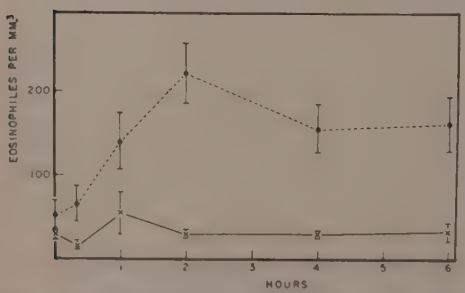

FIG. 1. Mean values and stand. errors of eosinophile responses of sensitized (– – – –) and normal (———) guinea pigs to 6 μg Ea N.

Discussion. That eosinophilia may occur in anaphylaxis independently of active antibody formation is established.‡ This suggests that the stimulus for the eosinophilia is to be found in a product released by the antigen-antibody reaction, or in one of the enzymes activated by the reaction(6). Particularly suggested is histamine itself, which has been reported to produce an eosinophilia in the guinea pig(2,7).

The finding of an eosinophilia in unsensitized guinea pigs after the larger Ea dose recalls the reports by Vaughn(7,8) that eosinophilia may be produced in normal guinea pigs by the injection of ascaris polysaccharide. However, the proper interpreta-

‡ After the conclusion of this study, we became aware of a comment by Campbell (Discussion, reference 5) that he had observed eosinophilia in passive, as well as in active anaphylaxis. The present paper, therefore, confirms and extends his earlier finding.

TABLE III. Blood Eosinophile Response to Injection of Large (160 μg) Doses of Egg Albumin Nitrogen in 4 Normal Guinea Pigs.

Route	Time in hr 0	¼–½	1	2	5	6
I.V.	56	53		175	175	259
"	118	109		68	156	237
I.P.	188	255	350	693	394	160
S.C.	55	33	49	222	316	38

tion of this finding is not clear. The claim (8) that the polysaccharide, independent of immune reaction to it, is primarily toxic and a stimulus to histamine release is not fully supported by the evidence, though this interpretation is certainly possible. Numerous instances have been described both in man and in the experimental animal, wherein antibody to various antigens may be "naturally" acquired. In man, well-documented instances would include the "natural" isoagglutinins and "normal" antidextrans(9). It would be difficult to prove that such "natural" antibodies did not play a role in the above instances. Further, since relatively large doses of antigen have been required for the production of eosinophilia in normal animals the possibility exists that such antibodies might be antibodies to antigen impurity(4). Therefore the experiments in which eosinophilia has been observed in normal animals may need reinterpretation, and particularly is this indicated in those cases(8) in which the animals were noted to die in convulsions, or with dyspnea, after the eosinophilia-producing injections. Such symptoms were not seen in the animals reported in Table III.

Summary and conclusions. It has been shown that eosinophilia in anaphylaxis is not dependent upon active antibody formation by the shocked animal. The development in several instances of a mild eosinophilia following the injection of relatively large doses of Ea into normal guinea pigs was noted.

1. Campbell, A. C. P., Drennan, A. M., and Rettie, T., *J. Path. and Bact.*, 1935, v40, 537.

2. Samter, M., *Blood*, 1949, v4, 217.

3. Bennette, I. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 772.

4. Vaughan, J. H., and Kabat, E. A., *J. Exp. Med.*, 1953, v97, 821.

5. Campbell, D. H., *J. Infect. Dis.*, 1943, v72, 42.

6. Burdon, K. L., *Mechanisms of Hypersensitivity and Their Significance In Relation to Certain Aspects of the Radiation Syndrome: A Critical Review*. Project Rep. USAF School of Aviation Med., Randolph Field, Texas, 1953.

7. Vaughn, J., *Blood*, 1953, v8, 1.

8. ———, *J. Path. and Bact.*, 1952, v64, 91.

9. Kabat, E. A., and Berg, D., *J. Immunol.*, 1953, v70, 514.

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Role of Hyperkalemia in Production of Ventricular Fibrillation Following Hypercapnia.* (22021)

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Rapid return to air breathing following 2 to 4-hour periods of high CO_2 breathing regularly produces hypotension and cardiac arrhythmias in dogs, and may produce ventricular fibrillation and death(1). Recently Sealy, Young, and Harris(2,3) have confirmed these results and have extended them in an attempt to determine the cause of the severe cardiac effect of this rapid fall in CO_2 tension and hydrogen ion concentration. They were impressed, as we were, with the similarity between the electrocardiograms recorded in the immediate post-hypercapnic period and those characteristic of hyperkalemia. These inves-

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tigators found a rise in serum potassium during hypercapnia which they believe is causally related to cardiac response.

Experiments reported in this paper suggest that the elevated plasma potassium is not sufficient of itself to produce the severe cardiac effects, but that a rapid decrease in arterial blood CO_2 tension and hydrogen ion concentration in the presence of elevated sub-lethal plasma potassium concentrations may produce cardiac arrest or ventricular fibrillation.

Methods. Five groups of experiments were carried out on dogs anesthetized with either sodium pentobarbital (Group 3) or sodium Pentothal (Groups 1, 2, 4, 5) administered intravenously. Blood pressures were recorded from a femoral artery by means of a strain gauge and one channel of a 2-channel direct writing oscillograph. Electrocardiograms were recorded on the second channel of the oscillograph using limb leads. A catheter for obtaining blood samples was passed through the femoral artery into the aorta and tied in place. Gas mixtures were administered through either an endotracheal tube or a tracheotomy tube from an open system.

In experiments in which KCl solution was administered, it was infused as a .16 M solution in an intravenous drip at approximately 100 drops per minute. Potassium determinations were carried out on a protein free filtrate of plasma with a flame photometer. Syringes for drawing 6 ml blood samples were prepared by allowing 0.1 ml of heparin solution containing 1 mg of heparin to dry overnight in each syringe in a warm oven.

Group 1. Thirteen dogs breathed 30% CO_2-70% O_2 for 2 hr followed by 40% CO_2-60% O_2 for an additional 2 hr, and were then returned to air breathing. Blood samples for assay of plasma potassium concentrations were drawn before starting the CO_2 breathing, at intervals during the 4 hours of CO_2 inhalation, and at intervals during the first hour after returning the animals to air breathing.

Group 2. Eleven dogs were administered KCl solution, while breathing air, until cardiac arrest or ventricular fibrillation appeared. Blood samples were drawn and electrocardiograms and blood pressures recorded before starting the KCl infusion and at intervals during infusion.

Group 3. Control blood samples and recordings were obtained on 8 animals who were then given 30% CO_2 in oxygen to breathe. After 10 minutes on this mixture an intravenous drip of KCl solution was started with the dogs continuing to breathe 30% CO_2. This was continued until ventricular fibrillation or cardiac arrest appeared.

Group 4. Eight dogs were given 30% CO_2 for 2 hours followed by 40% CO_2 for 2 hours and then while they continued to breathe 40% CO_2 an intravenous drip of KCl was started and continued until the heart stopped or the ventricles fibrillated.

Group 5. Thirty % CO_2 for 15 minutes followed by 40% CO_2 for 10 minutes was administered to 6 dogs. While the animals continued to breathe 40% CO_2, KCl solution was given as an intravenous drip until plasma potassium concentration was thought to be about 8 to 9 mEq/L. as judged by the amount of KCl given and by the electrocardiograms. This usually required 5 to 8 minutes. At this point the dog was changed from the high CO_2 mixture to 100% oxygen and hyperventilated at a rate of 50 breaths per minute and a positive pressure of 20 cm of water by means of a positive pressure respirator.

In all experiments during administration of KCl solution, blood pressures and electrocardiograms were recorded at intervals of one

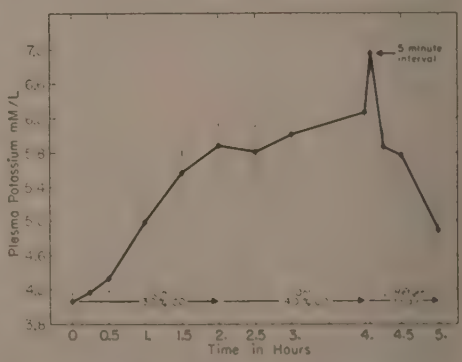

FIG. 1. Mean plasma potassium concentrations in 13 dogs before, during and following 4 hr of breathing 30% and 40% CO_2 in oxygen. Vertical bars extend one stand. error on each side of the mean.

TABLE I. Influence of Inhalation of High CO_2 Concentrations on Terminal Plasma Potassium Concentrations.

Group		No. of dogs	Plasma potassium conc., mEq/L Control	Before drip	Final
2	Breathing air	11	3.62 ± .132*		14.39 ± .329
3	Breathing 30% CO_2 for 15 min.	8	3.39 ± .090	3.66 ± .131	12.98 ± .572
4	Breathing 30% CO_2 for 2 hr followed by 40% CO_2 for 2 hr	8	3.84 ± .241	7.10 ± .416	13.79 ± .890

* Stand. error of mean.

to 2 minutes until severe cardiac arrhythmias appeared, and then continuously until cardiac arrest or fibrillation occurred. Blood samples were drawn at 5 minute intervals during the first few minutes of KCl infusion and at shorter intervals during the remainder of the experiment. An attempt was made to draw the final sample just before cardiac arrest took place. By watching the electrocardiogram and blood pressure tracing this was usually accomplished successfully.

Results. Fig. 1 presents the plasma potassium concentrations of 13 dogs in Group 1 during and following 4 hours of hypercapnia. Two of these dogs died in ventricular fibrillation after 5 minutes of air breathing and are not represented in the data after that time. A gradual increase in plasma potassium concentration during the hypercapnia was a regular finding. In 12 of 13 cases a further and more rapid increase took place during the first 5 minutes of air breathing. Immediately thereafter the concentration began to decrease but was still somewhat elevated one hour after the dogs had been returned to air breathing. The highest value recorded in any of these animals was 8.2 mM/L. at 5 minutes of air breathing following the 4 hours of hypercapnia. This dog died in ventricular fibrillation within seconds after the blood sample was drawn.

To be sure the peak concentration was not being missed, blood samples were drawn at one or 2-minute intervals for 12 minutes following hypercapnia in 4 dogs. In every case the highest concentration was found between 4 and 6 minutes. Table I presents the data for Groups 2, 3, and 4. The mean plasma potassium concentration at cardiac arrest in the dogs breathing air (Group 2) was 14.39 mM/L. This value agrees well with those reported by others(4). When the dogs were made hypercapnic prior to starting the KCl drip (Group 3) cardiac arrest occurred when the plasma potassium concentration averaged 12.98 mM/L. In the 8 dogs that breathed high CO_2 mixtures 4 hours before the KCl infusion began, the average plasma potassium concentration at cardiac arrest was 13.79 mM/L. Although hypercapnia of this degree appeared to lower the plasma potassium concentration at which cardiac arrest appeared, the differences were not impressive and in no instance in any of these groups did arrest or ventricular fibrillation occur when the plasma potassium level was as low as 8.2 mM/L., the highest level found to occur spontaneously during the rapid fall in pCO_2 and hydrogen ion concentration following 4 hours of hypercapnia. In other words no overlap in the terminal plasma potassium values between Group 1 and Groups 2, 3, and 4 appeared.

The possibility still existed, however, that the hyperkalemia and a rapid rise in pH and/or fall in CO_2 tension might be the necessary conditions to produce cardiac arrest or ventricular fibrillation. In earlier work we had demonstrated many times that blood pH falls to approximately 6.7 when a dog breathes 40% CO_2 for 15 minutes to 30 minutes and that rapid reversal of this hyercapnic, acidotic state is not followed by cardiac arrest or fibrillation as it commonly is when the hypercapnia has lasted 2 or more hours. Thus it appeared that the rapid fall in CO_2 tension or rise in pH alone were not producing the untoward cardiac effects. The present data indicate that the plasma potassium level attained following 4 hours of hypercapnia was not sufficient of itself to produce this effect.

To determine whether the combination of a rapid fall in hydrogen ion concentration and pCO_2 in the presence of a sub-lethal level of plasma potassium would produce cardiac ar-

TABLE II. Effect of Rapid Reduction in pCO_2 in Presence of Elevated Plasma Potassium Concentration.

	Plasma potassium concentrations, mM/L				
Dog No.	Control	High CO_2, 25 min.	KCl drip, 5 min.	3 to 7 min. on O_2	Result
1	4.2	4.6	10.2	9.3	Ventricular fibrillation
2	3.9	3.9	8.7	8.4	" "
3	3.7	3.8	8.4	11.5	" "
4	3.6	4.0		6.3	Cardiac arrhythmia (dog lived)
5	4.2		10.0	10.0	Ventricular fibrillation
6	3.6		11.9	11.3	Cardiac arrest

rest or fibrillation, the experiments in Group 5 were carried out. The data for this group of experiments are given in Table II. In one dog the plasma potassium concentration during the hyperventilation was only 6.3 mM/L. The electrocardiogram on this dog showed cardiac arrhythmias but the dog did not die. In 3 other cases the potassium level when fibrillation began was high enough that the hyperkalemia alone might have produced the result, although the values were well below the average for the dogs that received KCl during the hypercapnia. In 2 dogs of this series ventricular fibrillation occurred during the first 7 minutes of hyperventilation at a time when the plasma potassium concentrations were 8.4 mM/L. and 9.3 mM/L. respectively.

Comment. The pattern of changes in plasma potassium concentration during inhalation of high CO_2 mixtures appears to be different in dogs from that which has been observed in cats. Cattell and Civin(5) in attempting to separate the hypoxic effect from the hypercapnia effect of asphyxia, administered 10% CO_2 to cats and found a marked but temporary rise in serum potassium. MacKay(6) followed up this work by administering 35% CO_2 to cats and obtained similar results. An increase amounting to 3 to 5 times the normal value appeared within 10 minutes after beginning the CO_2 administration and returned to normal in 30 minutes. In addition a second rise took place when the cats were returned to air breathing. The first of these increases but not the second was blocked by tying off the blood supply of the abdominal viscera.

In dogs there appears to be a gradual increase in plasma potassium level during 4 hours of high CO_2 breathing, with an increase in slope during the first 5 minutes of air breathing following the CO_2. This latter change is similar to that reported in cats. In order to be sure that very rapid changes were not appearing during the first few minutes of CO_2 breathing in the dog, blood samples were drawn at 5-minute intervals for the first 30 minutes in several animals. All of the values obtained fell between the control and 30-minute sample. The explanation for this difference between cats and dogs in mobilization of potassium is not available at present.

Young, Sealy, and Harris(3) found a simultaneous rise in cardiac muscle potassium and plasma potassium concentration in the rat following 2 to 4-hour periods of exposure to 35 to 45% CO_2. What changes occur in heart muscle potassium during the rapid shift in blood pH are not known. The work of Fenn and Cobb(7) would suggest that heart muscle potassium should fall at the time carbon dioxide tension is falling. They found a shift of potassium from frog muscle to blood when carbon dioxide tension was reduced. Young *et al.*(3), however, found the cardiac muscle potassium still elevated after rats were removed from an atmosphere of high CO_2 to air. It may be that changes in some other electrolyte are playing a role in the cardiac effect. We have found blood pH regularly falling to approximately 6.7 when the animal breathes 40% CO_2. On changing to air the pH rises above 7.2 in 5 minutes or less. It is possible that this rapid shift in hydrogen ion concentration itself is responsible for the altered response of the heart.

A post-hypercapnic phenomenon similar to that exhibited by dogs has been demonstrated in man(8,9) and it has been suggested that

certain unexplained post-surgical deaths may be due to a rapid reduction in CO_2 tension which has become elevated during anesthesia. It would be interesting to know what changes take place in plasma potassium concentration in the human during prolonged exposure to severe hypercapnia. It seems likely that it is elevated but it would be important to know how rapidly and to what extent, as a function of time and CO_2 concentration, since this would have a bearing on the susceptibility of the heart to fibrillation or arrest when the CO_2 tension is reduced rapidly.

Summary and conclusions. 1. Dogs regularly show an increase in plasma potassium concentration during breathing of 30% and 40% CO_2 mixtures. Immediately following return to air breathing, there is a further sharp rise in this concentration. This secondary rise reaches a peak after 5 minutes of air breathing. 2. Concentration of plasma potassium following 4 hrs of high CO_2 breathing is not as high as that which is necessary to produce fibrillation or arrest when KCl solution is administered as an intravenous drip to normal or acidotic dogs. 3. Rapid return to air breathing following 30 minutes of high CO_2 breathing does not produce cardiac arrhythmias, ventricular fibrillation, or cardiac arrest in dogs. However, when this rapid elevation in blood pH and/or fall in pCO_2 takes place in the presence of sub-lethal but elevated plasma potassium concentrations, cardiac arrest or ventricular fibrillation may occur. Although this does not provide an explanation of the mechanism involved, it appears that the combination of elevated but sub-lethal plasma potassium concentration and a rapid fall in CO_2 tension and/or hydrogen ion concentration, work together to produce the damage to the heart.

1. Brown, E. B., Jr., and Miller, F., *Am. J. Physiol.*, 1952, v169, 56.

2. Sealy, W. C., Young, W. G., and Harris, J. S., *J. Thor. Surg.*, 1954, v28, 447.

3. Young, W. G., Sealy, W. C., and Harris, J. S., *Surgery*, 1954, v36, 636.

4. Huggins, R. A., Breckinridge, C. G., and Hoff, H. E., *Am. J. Physiol.*, 1950, v163, 153.

5. Cattell, M., and Civin, H., *J. Biol. Chem.*, 1933, v126, 633.

6. MacKay, J., *Am. J. Physiol.*, 1947, v151, 469.

7. Fenn, W. O., and Cobb, D., *ibid.*, 1935, v112, 41.

8. Miller, F. A., Brown, E. B., Jr., Buckley, J. J., Van Bergen, F. H., and Varco, R. L., *Surgery*, 1952, v32, 171.

9. Buckley, J. J., Van Bergen, F. H., Dobkin, A. B., Brown, E. B., Jr., Miller, F. A., and Varco, R. L., *Anesthesiology*, 1953, v14, 226.

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Photometric Method for Estimation of Elastase Activity.* (22022)

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The presence of an enzyme in animal pancreatic extracts which solubilizes elastin was first described by Balo and Banga(1). The activity was measured gravimetrically and by Kjeldahl nitrogen. Using the same technics, Hall(2), and later Lansing *et al.*(3), confirmed the presence of this enzyme in extracts of hog pancreas. Since both methods are somewhat cumbersome, a simpler colorimetric procedure was devised; it permits the direct measurement of the amount of dyed elastin which goes into solution when the dyed substrate reacts with pancreatic extracts.

Material and methods. Substrate. Purified elastin was prepared by the Lansing, Alex and Rosenthal(4) modification of the method of Lowry *et al.*(5). The ligamentum nuchae of horses was freed of the thin investing fascia and then ground into small pieces in a meat

* This investigation was supported by the Jacob Schultz Memorial Research Fund.

grinder. This well-ground material was covered with methanol and refluxed over a hot water bath for one hour. The alcohol was decanted, the residue covered with ether and refluxed for another hour. The residue was allowed to dry, added to 0.1 N sodium hydroxide which had been preheated to 93-98°C and the mixture kept at this temperature for 45 minutes. The sodium hydroxide was decanted and the residue washed thoroughly with water. This elastin (20 g) was air-dried, ground to 40-mesh size in a Wiley mill, and suspended in an orcein solution composed of 1 g of orcein in 100 ml of 70% ethanol to which 1 ml of concentrated hydrochloric acid had been added. The stained elastin was then washed repeatedly with large volumes of 70% ethanol and distilled water until both the ethanol and the aqueous washings were color-free. The stained elastin was finally washed with 70% ethanol and the air dried residue ground in a mortar. Samples of this substrate incubated in an enzyme-free system, as described in the Procedure, gave no color change in the supernatant. Quadruplicate nitrogen determinations of this elastin preparation by the Conway micro-diffusion method(6) gave an average of 14.5%.

Buffers: Tris (hydroxymethyl) aminomethane buffer 0.2M, pH 8.8, prepared according to Gomori(7). This buffer will hereafter be referred to as Tris. Phosphate buffer 0.7M, pH 6.0, prepared according to Sörenson (8).

Enzyme Preparation: This was prepared by extracting 10 g Viobin† (defatted autolyzed pork pancreas) with 50 ml N/40 hydrochloric acid for 20 minutes on a magnetic stirrer at room temperature and centrifuging the preparation at 12,000 rpm in an International Model PR-1 high-speed refrigerated centrifuge. The residue was reextracted with another 50 ml N/40 hydrochloric acid for 20 minutes and recentrifuged. The pooled clear supernatant measured approximately 90 ml. This crude extract had a pH of about 5, and was adjusted to pH 8.8 with 1N sodium hydroxide before use. There was no appreciable loss of elastolytic activity by keeping this preparation in the refrigerator for several weeks. Using our assay procedure, 10 g of Viobin extracted with 100 ml N/40 hydrochloric acid yielded a total of 12,000 to 13,000 elastase units when one elastase unit is defined as the amount of enzyme activity required to solubilize 1 mg of elastin substrate in 20 minutes at 37°C under our experimental conditions.

Procedure. Twenty mg portions of the dyed substrate were placed in small test tubes (13x125 mm) and 1 ml of Tris buffer, pH 8.8, 1 ml water, and 1 ml of suitably diluted enzyme preparation adjusted to pH 8.8 added to each. The tubes were then stoppered, placed in the horizontal position in a mechanical shaking device, which was immersed in a constant temperature water bath at 37°C. The shaker moves at a uniform rate of 46 excursions per minute. At the end of 20 minutes the tubes were removed from the bath and 2 ml of 0.7M phosphate buffer, pH 6, added to each tube to stop the enzyme reaction. With this drop in pH the color of the reaction mixture changed from a bluish purple to a reddish purple. This mixture was then centrifuged and the per cent transmission of the supernatant read on a Model DU Beckman Spectrophotometer at 590 mu. The results were then converted into specific activity, *i.e.*, elastase units per milligram enzyme preparation used.

Standard curve: Duplicate portions of the substrate, ranging from 5 mg to 25 mg, were placed in the test tubes. To each tube 1 ml of 0.2M Tris buffer, 1 ml water and 1 ml of enzyme preparation were added and the mixture incubated as described until all the elastin went into solution. This process took from 24 to 48 hours, depending on the strength of the enzyme preparation used. Then 2 ml of the phosphate buffer and 5 ml of water were added, giving a final dilution of 1:10. In all tubes a very small amount of insoluble material remained and this was attributed to non-elastin contamination of the substrate. This insoluble residue was estimated by nitrogen determination and gave an average of 3.8%.

Results. Following centrifugation the du-

† Viobin was generously contributed by the Viobin Corp., Monticello, Ill.

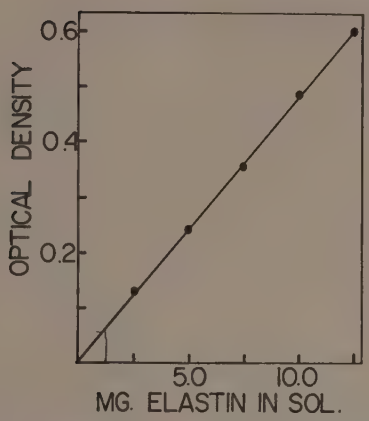

FIG. 1. Relationship between mg elastin dissolved and optical density (590 mu) of the orcein dye liberated.

plicate samples were read at 590 mu on the Beckman Spectrophotometer and the readings plotted as optical density vs mg elastin per 5 ml solution (Fig. 1). The duplicate determinations were in good agreement. Regardless of the analytical procedure, it would seem that standardization of a new elastin preparation would always be indicated, since there may be slight variation in the constituents of any given batch, and there is no reason to assume that any of these preparations represents pure elastin.

Time curve. By using a stop watch, tubes containing the substrate, buffer, water and enzyme mixture, as described, were withdrawn from the water bath at intervals and the reaction stopped by the addition of the phosphate buffer. Results were recorded as time in minutes vs milligrams elastin in solution (Fig. 2).

Dilution curve. Dilutions of a lyophilized enzyme preparation, varying from 0.2 to 5.0 mg elastase powder per ml, were used, with an incubation time of 20 minutes. The results were plotted as milligrams dissolved elastin vs enzyme concentration, and they followed a zero order reaction when less than 7 mg or 35% of the 20 mg substrate used was solubilized (Fig. 2).

pH Optimum. Hall and Gardiner(9) showed that the pH optimum of elastolytic activity lies between 8.7 and 9.2, with a different peak for substrates of different animal origin. On horse ligamentum nuchae, prepared as described, the pH optimum for our enzyme preparation lies between 8.7 and 9.4. The inadequacy of the 0.1N carbonate buffer, pH 10.23, used by Balo and Banga, was verified by us, using the colorimetric method. The pH after incubation dropped to levels varying from 9.8 to 8.9. Buffers of sufficient molarity to hold the pH of the reaction mixture at 10.23 gave minimal elastolytic activity.

Comparison of methods. Different amounts of the elastin substrate were weighed on an analytical balance and exposed to the enzyme buffer mixture until varying amounts of the elastin had gone into solution. The supernatant was removed after centrifugation and the amount of solubilized elastin determined by the colorimetric method described. The nitrogen content was assayed by the Conway micro-diffusion method, using 1 ml of the supernatant. Appropriate controls consisting of identical mixtures of the enzyme buffer solution were also assayed for their nitrogen content. For the gravimetric procedure the residues were oven-dried at 100°C and weighed on an analytical balance, the amount of elastin in solution being the difference between the original weights and that of the residues. The results of all three methods are recorded in Table I.

Discussion. The assay procedure described

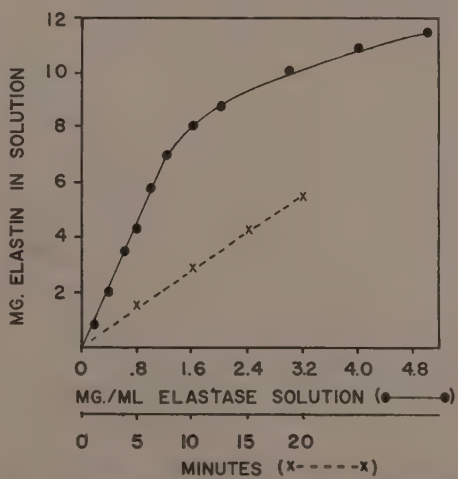

FIG. 2. Effect of time and of elastase concentration on dissolution of elastin.

TABLE I. Milligrams Elastin Dissolved by Action of Elastase as Measured by:

Gravimetric procedure	Colorimetric method	Total nitrogen in solution
21.8	21.8	21.3
21.3	22.7	25.6
19.7	20.2	19.3
17.9	18.2	17.8
18.0	17.5	17.6
10.1	10.9	11.1
8.9	7.9	8.2
12.5	12.5	12.7
11.0	11.6	11.1
4.5	3.7	4.8
3.0	2.9	2.4
5.1	4.3	5.9
18.8		17.8
18.0		19.1
26.8		26.0
33.0		33.1
	19.9	19.5
	3.4	2.4
	11.2	12.7
17.9		17.3
11.8		11.0
19.7		18.5

here has the advantage of rapidity, simplicity and reproducibility, as well as the production of a visible reaction, so that one gauges at a glance whether activity is present and to what degree, without first having to complete the procedure. Hall(10) assayed the enzyme preparation by incubation for 18 hours, without agitation. It would appear that one cannot determine with accuracy the specific activity of the enzyme preparation under conditions where the dissolution of elastin is no longer linear. The risk of bacterial contamination is reduced by using the shorter incubation period in our method. No inactivation of the enzyme was noted by the amount of agitation used by us; instead, it was found that the enzymatic activity was increased up to 100% as compared to incubation without agitation.

When the same enzyme solution is tested against batches of horse ligamentum nuchae substrate prepared at different times, the activity changes. This is most probably a result of variation in particle size and in the amount of dye absorbed by the elastin. This variable may be cited as a disadvantgae of the method, but standardization of the substrate each time a new batch is prepared would appear to be necessary, irrespective of the analytical procedure used. The problem can be minimized by preparing larger quantities of the substrate each time.

Summary. A colorimetric method for the estimation of elastase activity has been presented. When dyed elastin substrate prepared from horse ligamentum nuchae is used, the pH optimum lies between 8.7 and 9.4, and the reaction velocity is linear only when less than 35% of the elastin is dissolved. When compared with the gravimetric and nitrogen procedures, this method has the advantages of rapidity and simplicity.

1. Balo, J., and Banga, I., *Schweiz. Z. Path.*, 1949, v12, 350; *Biochem. J.*, 1950, v46, 384.

2. Hall, D. A., *Nature*, London, 1951, v168, 513.

3. Lansing, A. I., Rosenthal, T. B., Alex, M., and Dempsey, E. W., *Anat. Rec.*, 1952, v114, 555.

4. Lansing, A. I., Alex, M., and Rosenthal, T. B., *J. Gerontology*, 1950, v5, 112.

5. Lowry, O. H. Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, 1941, v139, 795.

6. Conway, E. J., *Microdiffusion Analysis and Volumetric Error*, Van Nostrand Co., Inc., London, 1950, 3rd Ed.

7. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 33.

8. Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, v12, 393.

9. Hall, D. A., and Gardiner, J. E., *Biochem. J.*, 1955, v59, 465.

10. Hall, D. A., *ibid.*, 1955, v59, 459.

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Comparative Effects of Anesthetic Agents on Incidence of Ventricular Fibrillation During Hypothermia. (22023)

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Cardiac ventricular fibrillation and asystole are two factors which have produced a limit for experimental and clinical hypothermic procedures. Fibrillation has generally occurred at higher temperatures than asystole. Elimination of factors which tend to cause fibrillation might therefore permit the use of lower temperatures for such procedures. Choice of anesthetic agent is conceivably involved in the production of fibrillation. Available data are on the whole inconclusive as to the role that various anesthetic agents play, although Covino *et al.*(1) state that ventricular fibrillation occurs less frequently in dogs receiving thiopental or ether than in those receiving pentobarbital.

It therefore seemed desirable to study the effects of commonly used agents, ether, cyclopropane and pentobarbital with regard to their effect on production of ventricular fibrillation or asystole during hypothermic anesthesia.

Methods. Healthy mongrel dogs of both sexes ranging in age from 6 to 12 months and weighing between 3.6 and 9.1 kg were used. No premedication was given. Eleven dogs were anesthetized with cyclopropane and 10 anesthetized with ether. They were intubated as soon as depth of anesthesia was sufficient to permit it. They were connected to a closed to-and-fro CO_2 absorption system with controlled hyperventilation at 50 ± 5 respirations per minute. The hair was removed from body, neck and proximal parts of legs. A rectal thermometer was inserted at least 10 cm. Needle electrodes for Lead II of electrocardiograph were attached. The animals were immersed on their sides in ice bath at 1 to 3°C with only head and part of neck elevated above bath. The level of anesthesia was maintained so lateral canthus reflex was lost and medial canthus reflex present. This provided anesthesia which would be adequate for surgical procedures. When rectal temperature reached 22°C artificial respiration with 100% oxygen was continued with a controlled phase respirator regulated at 50 ± 5 respirations per minute. A third series of dogs was given 30 mg per kilo of pentobarbital intravenously and intubated as soon as possible. Hair was removed, rectal thermometer inserted, needle electrodes attached and animals were at once connected to a control phase respirator using 100% oxygen at 50 ± 5 respirations per minute and immersed in ice bath. Time required for cooling was recorded. Electrocardiographic tracings, rectal temperatures and pulse were noted continuously and records made at 5-minute intervals or less. No surgical procedures were carried out.

Results. The pulse diminished approximately 12 beats per minute for each degree C of cooling of dogs down to approximately 16°. At lower temperatures, the rate of slowing could not be correlated with rate of cooling. Variable periods of asystole occurred when the pulse was 10 per minute or less and only by electrocardiographic observations could the heart be determined to still be active.

As indicated in Table I, 8 of 11 dogs receiving cyclopropane went into ventricular fibrillation (average temp. 19.6°), 2 of 10 dogs on ether fibrillated (average temp. 18.9°), while none of the dogs receiving pentobarbital showed ventricular fibrillation. As might have been expected, animals that fibril-

TABLE I. Terminal Cardiac Event in Dogs Receiving Different Anesthetic Agents.

	Cyclopropane	Ether	Pentobarbital sodium
No. of dogs	11	10	10
Asystolic deaths	3	8	10
Mean rectal temp., °C	9.3	12.0	11.3
Mean cooling time, min.	107	100	110
Ventricular fibrillation	8*	2†	0‡
Mean rectal temp., °C	19.6	18.2	
Mean cooling time, min.	63	54	

*-† $p < .001$ Significant
*-‡ $p < .05$ "
†-‡ $p > .05$ Not significant

lated died at higher temperatures than those which died of asystole. There were considerable differences in muscular activity of the 3 groups of animals. Animals receiving cyclopropane tended to be relatively active even though eye signs indicated that a stage of surgical anesthesia had been reached. Gross movements and even respiratory efforts appeared when the cyclopropane was gone at temperature below 28°. The animals that received ether showed some muscular action at temperatures below 28° but these movements were not marked. Animals receiving pentobarbital were quiet and showed no shivering or other muscular action after being cooled.

The 3 animals in the cyclopropane group dying of asystole expired at a mean temperature of 9.3°, the 8 asystolic ether deaths occurred at an average temperature of 12° while the asystolic pentobarbital deaths occurred at an average of 11.3°.

Discussion. It has been pointed out that an anesthetic agent or relaxing agent is necessary during the early phase of cooling to prevent shivering or other protective movements by the dog which inhibit body cooling. Haterius and Maison(2) suggested that the ideal anesthetic agent should control muscle activity and shivering until cold narcosis itself was sufficient for such control. They stated that cyclopropane was valuable an an anesthetic agent but were indefinite as to the number of animals used with cyclopropane or the average temperature to which they could be cooled successfully. One dog survived cooling to 11.7°. They also reported that dogs cooled during ether anesthesia could withstand cold better than those receiving sodium pentobarbital(3). Artificial respiration was given "as needed." In studies on effect of age on cooling, Maguire and Merendino(4) observed that adult dogs receiving 60 mg/kg of sodium pentobarbital died at average temperature of 20.4°. Eleven of these 15 died of ventricular fibrillation. Using young dogs, the average temperature of death was 12.6°, all from asystole. It appears that both youth and lack of occurrence of ventricular fibrillation influenced temperatures of death. Covino *et al.* (1) observed that 16 out of 19 dogs anesthetized with pentobarbital died of ventricular fibrillation. Those results would seem perhaps different from ours. No information, however, was given as to age of their animals. Judging from the weights (6.7 to 17.5 kg) it might be assumed that their dogs were older animals. Covino's group used push-pull artificial respiration with room air only below 26 to 24°C while our animals received hyperventilation with oxygen continuously. Our experiments, those of Maguire and Merendino(4), and of Covino *et al.*(1) all indicate that the temperature of animals dying of ventricular fibrillation is higher than that of those whose hearts have an asystolic termination of action.

Animals receiving pentobarbital undoubtedly have a considerable amount of the drug left in their bodies at the time of hypothermic deaths. Animals receiving ether probably have most of it eliminated when death occurs, but due to high solubility of ether in water and the progressively decreased circulation in hypothermic animals, some of the ether is undoubtedly left in the system. Cyclopropane is much more volatile than ether and is less soluble in water. It therefore may be assumed to be almost entirely gone from tissues by the time that cold narcosis has appeared. Anesthetization with cyclopropane followed by its practical elimination might be expected to diminish the tendency to ventricular arrhythmias, if the concept that deeper cyclopropane anesthesia increases the incidence of such irregularities(5) is accepted. Efficient concomitant elimination of carbon dioxide by assisted respiration is also said to prevent or abolish such arrhythmias(5). The use of cyclopropane, however, did not afford protection against ventricular fibrillation in the hypothermic dog, although those animals receiving cyclopropane which did not fibrillate reached the lowest temperatures of all those observed. The results obtained indicate that sodium pentobarbital may have had a protecting effect on the cardiac irritability while cyclopropane showed no such effect. The effects of ether were somewhat intermediate between those of the other two agents.

Summary. The terminal cardiac events of 31 hypothermic dogs, 6 to 12 months old, were observed. Eleven animals received cy-

clopropane and 10 each received ether and pentobarbital sodium as the pre-hypothermic anesthetic agents. All animals were hyperventilated with oxygen after intubation until the experiments were terminated. Rectal temperatures were continuously observed and electrocardiograms were taken at frequent intervals as the dogs were cooled until cardiac activity ceased. No surgical procedures were carried out. Eight of 11 animals receiving cyclopropane, 2 of 10 receiving ether, and none of 10 receiving pentobarbital died in ventricular fibrillation. The mean rectal temperature of animals on cyclopropane or ether dying of ventricular fibrillation was 19°C. The mean rectal temperatures of all the deaths after the use of cyclopropane was 16.7°, after ether, 13.4°, and after pentobarbital sodium, 11.3°.

1. Covino, B. G., Charleson, D. A., and D'Amato, H. E., *Am. J. Physiol.*, 1954, v178, 148.

2. Haterius, H. O., and Maison, G. L., *ibid.*, 1948, v152, 225.

3. Hegnauer, A. H., D'Amato, H., and Flynn, J., *ibid.*, 1951, v167, 63.

4. Maguire, R. X., and Merendino, K. A., *Arch. Surg.*, 1955, v70, 367.

5. Goodman, L. S., and Gilman, A., *The Pharmacological Basis of Therapeutics*, 2nd Ed. (The Macmillan Co., New York) 1955, 87.

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Effect of Malonate and Antimycin A on Renal Tubular Transport of p-Aminohippurate.*† (22024)

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The dependence of renal tubular transport systems on certain energy producing reactions might be established by the use of specific metabolic inhibitors. There exists, for example, a correlation between the capacity of certain chemical compounds to inhibit the oxidation of succinic acid *in vitro* and to decrease the renal tubular excretion of p-aminohippurate (PAH) and phenolsulfonphthalein (PSP) *in vivo*(1). In the present report additional data are presented to support the hypothesis of Shideman and Rene(1) that energy derived from succinate oxidation (and Krebs cycle oxidations) is involved in the renal tubular mechanism which effects the excretion of PAH.

Materials and methods. Female, albino rats (150-200 g) of the Holtzman strain were anesthetized by the intraperitoneal administration of 35 mg/kg of pentobarbital and the right (control) kidney was removed. Cortical slices from this kidney were prepared and approximately 100 mg of these slices were placed in 20 ml beakers containing a volume of 2.7 ml of the medium employed by Cross and Taggart(2). The concentration of PAH, however, was half that used by these authors. The

TABLE I. Effect of Malonate Administration on Ability of Renal Cortical Slices of Rat to Accumulate PAH. Each animal received 1.2 ml of M sodium malonate subcutaneously 2 hr before and 0.6 ml of the same solution one hr before removal of the left kidney (6 animals).

	S/M concentration		
Rat No.	Before malonate (right kidney)	After malonate (left kidney)	% inhibition
1	8.42	2.43	80.8
2	7.81	3.59	62.0
3	6.07	2.27	75.0
4	8.39	4.07	58.5
5	10.10	8.19	21.0
6	14.90	6.27	62.1
	Mean ± S.E.		59.9 ± 8.5
			P <.01

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† A preliminary report was presented at the 37th annual meeting of Fed. of Am. Soc. for Exp. Biol., Chicago, Ill., April, 1953.

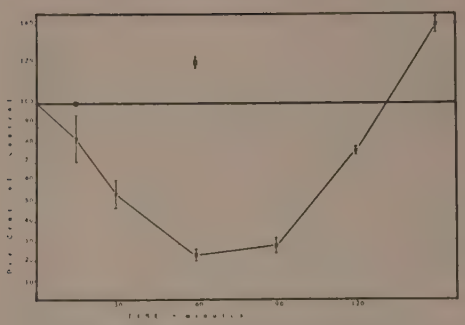

FIG. 1. Impairment of PAH accumulation by renal cortical slices from rats following administration of 1 g/kg sodium malonate. Each point with its standard error represents the data obtained from 6 animals. ×—malonate, □—saline, isosmotic with and equivalent in amount to malonate administered.

beakers were incubated for one hour at 25°C in a Dubnoff Metabolic Shaking Incubator with continuous oxygenation. Following incubation, the slices were removed from the medium, weighed, and the concentration of PAH in the slice and medium determined. The capacity of the cortical slices to accumulate PAH was expressed as the ratio of the concentration of PAH in the slice to that in the medium (S/M ratio). Following removal of the right kidney, as mentioned above, sodium malonate or antimycin A‡ was administered either subcutaneously, intraperitoneally, or intravenously and at varying periods of time thereafter the left kidney was removed from each of the test animals and the capacity of cortical slices to accumulate PAH was determined. Antimycin A was dissolved in either polyethylene glycol 200 or propylene glycol for injection and in 95% ethyl alcohol for addition to the medium in the *in vitro* experiments.

Results. Following subcutaneous administration of sodium malonate in the dosage indicated in Table I there is a significant decrease (59.9%) in capacity of the kidney to concentrate PAH. Dosage schedule of sodium malonate in this representative experiment is identical with that found by Busch and Potter(3) to inhibit *in vivo* metabolism of succinate by rat kidney. In other animals which received an equivalent amount of saline, isosmotic with malonate solution employed, there was no significant alteration in the capacity of cortical slices to accumulate PAH.

The reversible nature of the inhibitory effect of malonate on transport of PAH is apparent from the data presented in Fig. 1. Sixty minutes after administration of malonate the peak effect was observed. Inhibition of this renal excretory mechanism was not demonstrable for more than approximately 2 hours after administration of malonate. Two and one-half hours following administration of the inhibitor, there was noted an appreciable increase in capacity of renal cortical slices to accumulate PAH. Intravenous saline (isosmotic with malonate) produced no inhibition 15 minutes after administration. Sixty minutes after saline, however, there was observed a slight increase in the capacity of renal cortical slices to accumulate PAH. Since the S/M ratios were based on wet weight of renal tissue, the possibility existed that this slightly enhanced capacity to accumulate PAH might be due to alteration in water content of the kidney following injection of hypertonic saline. However, no significant differences in dry weights of renal slices before and after intravenous injection of hypertonic saline were found.

The influence of varying the dosage of malonate, administered intravenously, is illustrated in Fig. 2. At doses between 0.6 and 1.0 g/kg a linear relationship was found to exist between the intravenous dose of malonate

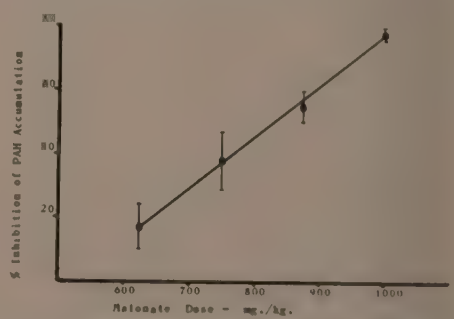

FIG. 2. Relationship between dose of sodium malonate and inhibition of PAH accumulation by renal cortical slices of the rat.

‡ Kindly supplied by Dr. F. M. Strong, Department of Biochemistry, University of Wisconsin, Madison.

TABLE II. Inhibitory Effect of Antimycin A on Accumulation of PAH by Renal Cortical Slice of the Rabbit. Slices were prepared from cortical portion of the kidney and incubated for one hr. Antimycin was dissolved in 95% alcohol and added in a volume of 0.1 ml to each flask. Slices incubated in a medium containing the same amount of alcohol but with no antimycin served as controls.

μg antimycin A/g tissue	% inhibition
2	5.8
4	13.5
6	43.8
8	63.5
10	68.5
100	82.6
1000	91.1
1500	97.4

and percentage inhibition of uptake of PAH.

Addition of antimycin A to a medium containing slices of renal cortex interferes with their capacity to concentrate PAH (Table II). These data are from a representative experiment in which the rabbit was employed to obtain sufficient tissue to determine the effects of a fairly wide range of concentrations of the inhibitor. Similar findings obtained in the rat. The succinoxidase activity of rat kidney is inhibited by approximately 2 to 7 μg of antimycin A per gram of tissue(4). In the same concentration range, antimycin A also inhibits the accumulation of PAH by renal slices of the rabbit.

In Table III are shown the effects of parenteral administration of antimycin A on accumulation of PAH by renal slices obtained from animals treated as described below. Administration of antimycin A produced marked inhibition of PAH uptake. Reif and Potter (5) reported that comparable doses significantly suppress succinoxidase activity in kidney of the rat. Animals receiving only propylene glycol intraperitoneally or 0.5% saline and polyethylene glycol 200 intravenously served as controls. These procedures produced no significant effect on capacity of cortical slices prepared from such animals to accumulate PAH. Administration of saline prior to the polyethylene glycol 200 was effective in preventing small but significant effects produced by the latter compound when it was injected intravenously.

Discussion. In previous studies metabolic inhibitors have been used to establish the dependence of a given physiological function on specific energy yielding reactions. 2,4-Dinitrophenol (DNP), a compound which is believed to uncouple aerobic oxidative and phosphorylation reactions, has been shown by Taggart and Forster(6), and Mudge and Taggart(7) to inhibit the renal tubular excretion of PAH, diodrast, and phenol red. Metabolic blockade at the level of the Krebs cycle, with either dehydroacetic or malonic acids(1) or inhibition of oxidative phosphorylation by DNP(7), does not indiscriminately influence all renal tubular functions. These observations suggest that there is some specificity in the allocation of energy derived from oxidative mechanisms and breakdown of phosphate bond compounds for the various renal tubular functions. Although metabolic inhibitors might produce a specific biochemical lesion *in vitro*, the interpretation that their *in vivo* action is a reflection of their *in vitro* effect might be questioned. Sodium malonate and antimycin A were chosen because of the evidence for correlation of *in vivo* and *in vitro* inhibitory effects. Busch and Potter(3) have presented evidence for the *in vivo* inhibition of succinate metabolism by malonate. The same concentration of antimycin A which Reif and Potter(5) have found to inhibit succinoxidase activity in the kidney, also produces an inhibitory effect upon the renal transport of PAH. Therefore, our data, correlated with those of the above authors, are highly suggestive that succinate oxidation (and Krebs cycle oxidations) functions *in vivo* as a source of energy for this transport mechanism.

Availability of energy derived from the oxidation of malonate for the transport of PAH

TABLE III. Effect of Antimycin A Administration on Accumulation of PAH by Renal Cortical Slice of the Rat. Following removal of control (right) kidney,(a) antimycin A (1 mg/cc in propylene glycol) was administered, after 40 min. the left kidney was removed or(b) 4 cc/kg of 5% saline was administered, followed in 30 min. by intravenous injection of antimycin A (1 mg/cc in polyethylene glycol 200) 15 min. after which the remaining kidney was removed.

Route	No. of animals	Dose, mg/kg	Inhibition of PAH accumulation (% ± S.E.)
Intraper.(a)	6	3	63.4 ± 10.5
Intraven.(b)	6	1	91.4 ± 1.5

has to be considered as a possible explanation for the capacity of the renal slice to accumulate the latter material 2½ hours after the administration of malonate. Busch and Potter (3) were able to recover only part of the malonate from the urine of animals receiving this compound. It is known that malonate can be oxidatively metabolized *in vivo*(8,9) and, therefore, may contribute to the pool of metabolites from which the excretory mechanism for PAH derives its energy. Furthermore, it is possible that malonic acid is metabolized or removed from the cell more rapidly than is succinate. Succinate (and other members of the Krebs cycle) which accumulate in the presence of malonate would be expected to be present in amounts greater than are usually found and supposedly would provide an additional source of energy for the transport of PAH. Whether or not either of these proposed mechanisms is correct remains to be determined.

Summary. The renal tubular excretion of PAH in the rat is inhibited by the *in vivo* administration of sodium malonate or antimycin A. The data here presented, when correlated with the results of other investigators, strongly suggest succinate oxidation can provide an *in vivo* source of energy for that renal mechanism which is responsible for the tubular excretion of PAH.

1. Shideman, F. E., and Rene, R. M., *Am. J. Physiol.*, 1951, v166, 104.

2. Cross, R. J., and Taggart, J. V., *ibid.*, 1950, v161, 181.

3. Busch, H., and Potter, V. R., *J. Biol. Chem*, 1952, v198, 71.

4. Potter, V. R., and Reif, A. E., *ibid.*, 1952, v194, 287.

5. Reif, A. E., and Potter, V. R., *Cancer Res.*, 1953, v13, 49.

6. Taggart, J. V., and Forster, R. P., *Am. J. Physiol.*, 1950, v161, 167.

7. Mudge, G. H., and Taggart, J. V., *ibid.*, 1950, v161, 173.

8. Lee, J. S., and Lifson, N., *J. Biol. Chem.*, 1951, v193, 253.

9. Lifson, N., and Stolen, J. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 451.

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Effects of Digitoxin on Exchangeable and Tissue Potassium Contents.* (22025)

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The effect of digitalis on both normal and abnormal myocardium is well known. Although it is an established fact that the glycosides have a direct effect on the heart, controversy still exists as to whether they have an *extracardiac* effect as well. Because the chemical structure of digitalis is similar to that of other steroid compounds which are known to affect salt and water metabolism—for example, sex and adrenal cortical hormones, it might be suspected that the glycosides would act similarly. There are, in fact, experimental evidences to suggest that these agents, under certain circumstances, act in a fashion similar to the adrenal cortical hormones(1,2).

A previous study(3) suggested that the intravenous administration of 0.2 mg of digitoxin per kg of body weight results in reversible shifts in the distribution of body fluids. The availability of radioactive potassium (K^{42}) has made possible the direct measurement of the exchangeable potassium con-

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tent in intact animals. The purposes of the present study were (1) to determine the effects of large daily doses of digitoxin (0.1 mg/kg) on the exchangeable potassium content in normal rabbits, and (2) to determine the effect of a single large dose of digitoxin (0.2 mg/kg) on the tissue potassium content.

Material and methods. Sixteen normal male adult rabbits of mixed breeds were used; they were kept in individual metabolism cages and were fed a stock diet of compressed pellets. Tap water was given without restriction. In order to acclimate the animals to their new environment, they were placed in the cages one week prior to the initiation of the baseline studies. Commercial crystalline digitoxin dissolved in a 40% solution of alcohol was employed. *Experiment 1.* Direct measurements of the exchangeable potassium content (Ke) were made with K^{42} at weekly intervals in 8 animals. Starting on the 8th day and continuing every day for a week, 0.1 mg of digitoxin per kg body weight was given intravenously to each animal. No digitoxin was given during the third week. The initial Ke determination was made on the first day of the experiment; the second, on the 7th day. In the analysis of the data, the mean of these two determinations was used as the baseline value for each animal. Subsequent Ke determinations were made at weekly intervals. *Experiment 2.* Each of 4 animals was given an intravenous injection of 0.2 mg of digitoxin per kg of body weight through a marginal vein immediately prior to the administration of K^{42}. Another group of 4 animals were not given digitoxin and served as controls. Twenty-four hours after intravenous injection of the radiopotassium, the animals were killed by exsanguination and analyses for radiopotassium and for total water content were performed on 18 different tissues.

Measurement of the exchangeable potassium content (Ke). The preparation of the potassium solution for injection has been previously described(4). Five ml of the potassium solution, containing 30 microcuries of K^{42} in 0.5 meq, was injected intravenously. The urine was collected for 24 hours; a spot specimen was then obtained by catheterization and its specific activity was determined. The following formula was used to calculate the value for the exchangeable potassium content of the body: $Ke = \frac{Ki^{42} - Ko^{42}}{Ku^{42}/Ku^{39}}$, in which Ki^{42} = quantity of radiopotassium administered (arbitrary units), Ko^{42} = quantity of radiopotassium excreted in the urine in 24 hours, Ku^{42} = concentration of radioactivity in the spot specimen, Ku^{39} = concentration of total inorganic potassium in the spot specimen, and Ku^{42}/Ku^{39} = specific activity of the spot specimen. The potassium concentration in the urine and in the serum was determined by flame spectrophotometry. *Tissue analyses.* Three portions (1 to 3 g) of each tissue were obtained. They were kept in covered Petri dishes and were weighed as rapidly as possible on a Gramatic balance to an accuracy of 0.1 mg. Two portions were digested in concentrated sulfuric acid for determination of the radiopotassium concentration. The digest was made up to a volume of 20 ml and was counted with a dipping tube and a scaling circuit. The tissue content of K^{42} was expressed in counts per minute per kg of tissue (wet weight). In the calculation of the *total tissue content* of potassium, it was assumed that at 24 hr the K^{42} was in equilibrium with the non-radioactive potassium in the body, and the specific activity of the spot specimen obtained at 24 hr (K^{42} (cpm/1)/K^{39} (meq/1)) was used to calculate the total potassium content in each tissue. This value was expressed in meq/kg. *Tissue water content* was determined on the third aliquot which was dried to a constant weight at 110°C for 18 to 24 hours. *Statistical method.* The significance of the difference between the means was determined by the "t" test(5). In the first experiment, each animal served as its own control. The differences between the baseline values and the values at the end of the second and third week were calculated for each animal; the mean difference between the baseline values and each of the subsequent determinations was then obtained. The significance of the mean difference was then tested. In the analysis of the results of experiment 2, the "t" test was again used to determine the significance of the difference between the group

TABLE I. Effects of Digitoxin in Doses of 0.1 mg per kg Daily for 7 Days on Exchangeable Potassium and Serum Potassium in 8 Normal Rabbits.

	Wt (kg)	Exchangeable potassium (meq)	Exchangeable potassium (meq/kg)	Serum potassium (meq/l)
Baseline values	2.578	207.4	78.7	5.35
Wk 1*	2.669‡	172.4‡	63.2‡	4.98‡
" 2†	2.769‡	232.9	83.7	5.29

* Mean values after 7 days of digitoxin.
† Mean values 7 days after discontinuation of digitoxin.
‡ Statistically significant difference when this figure is compared with the mean baseline value: P = less than 0.02.

means obtained in the test and control groups of animals. The calculated value for "t" was compared with the value at (n-l) degrees of freedom, as obtained from standard tables, and expressed as "P". A "P" value of 0.02 or less was considered significant.

Results. Experiment 1. (Table I). During the three-week period of observation, the mean body weight of the 8 animals in this group increased progressively from 2.578 to 2.769 kg. After 7 days of digitoxin the mean exchangeable potassium content decreased significantly from a baseline value of 207.4 meq (78.7 meq/kg) to 172.4 meq (63.2 meq/kg). The serum potassium concentration at this time was significantly lower than the baseline value, having fallen from 5.35 to 4.98 meq/1. One week after discontinuation of the drug, the exchangeable potassium content had risen to 232.9 meq.

Experiment 2. (Table II). All of the tissues in the rabbits given digitoxin contained less potassium than the tissues in the uninjected control animals; however, statistically significant differences in the mean potassium contents were observed only for the diaphragm, the abdominal muscle, and the subcutaneous connective tissue. No significant differences in the tissue water contents were found between the digitoxin-injected and the normal control animals and these values have not been included in the table.

Comment. The results of the first experiment indicate that the body store of potassium is considerably depleted within a week after the administration of digitoxin in daily doses of 0.1 mg/kg is begun. This reduction in the exchangeable potassium content was reflected in the slight, but significant, decrease in the serum potassium concentration. After discontinuation of the drug, the changes in exchangeable potassium content and in serum potassium concentration were promptly reversed.

The results of Experiment 2 suggest that the decrease in the total body content of potassium which follows the administration of a single dose of 0.2 mg of digitoxin per kg of body weight is due to a generalized loss of potassium from most of the tissues and organs of the body; the magnitude of this loss was greatest and most consistent in the abdominal and diaphragmatic muscles and in the subcutaneous connective tissue.

The results obtained in the present study are similar to those reported to occur during the administration of desoxycorticosterone acetate to normal animals(6,7). The prolonged administration of desoxycorticosterone may cause a decrease in the potassium content of muscle and necrosis of myocardial fibers. These lesions cannot be distinguished from those produced by the dietary restriction of potassium. A previous study in this laboratory has shown that the administration to nor-

TABLE II. Effects of a Single Dose of Digitoxin, 0.2 mg per kg, on Tissue Potassium Content in Normal Rabbits.

Tissues or organs	Potassium content (meq/kg) (mean ± S.E.) Uninjected control animals	Animals given digitoxin
Quadriceps muscles	66.77 ± 4.10	50.00 ± 6.06
Gastrocnemius muscle	66.42 ± 1.54	58.39 ± 4.75
Adrenal gland	63.33 ±24.60	37.42 ±22.80
Abdominal muscle	57.03 ± 4.21	33.34 ± 2.52*
Appendix	54.62 ± 4.10	42.28 ± 5.90
Liver	48.49 ± 3.33	38.69 ± 4.32
Spleen	45.63 ± 8.67	41.75 ± 5.77
Stomach	44.97 ± 5.18	34.79 ± 4.40
Diaphragm	44.19 ± 3.83	25.62 ± 3.76*
Heart, ventricle	41.63 ± 3.40	32.58 ± 3.34
Kidney	41.30 ± 3.30	31.91 ± 4.49
Lung	39.92 ± 2.07	29.97 ± 3.88
Heart, auricle	28.90 ± 3.41	19.59 ± 3.07
Subcut. connective tissue	28.09 ± 2.43	17.49 ± 1.86*
Skin	17.47 ± 2.99	11.07 ± 1.97

* Statistically significant difference when compared with the mean control value: "P" = 0.02 or less.

mal rabbits of large doses of cortisone acetate under experimental conditions closely resembling those employed in the present study also produces a significant decrease in the exchangeable potassium content(8). The results of the present study suggest that one of the effects of digitoxin, administered in the dosages and in the manner employed for these experiments, is to deplete the body store of potassium. This depletion is thought to be the result of an extracardiac effect of digitoxin.

Summary. 1. The daily intravenous administration of 0.1 mg of digitoxin per kg of body weight to normal rabbits resulted, within 7 days, in an increase in body weight and a reduction in the serum potassium concentration and the exchangeable potassium content. These changes in potassium metabolism were corrected within one week by discontinuing the glycoside. 2. The intravenous administration of a single large dose of digitoxin (0.2 mg per kg) to normal rabbits resulted within 24 hours in a decrease in the mean potassium content of all the tissues and organs. Statistically significant decreases in potassium content were noted in the abdominal and diaphragmatic muscles and in the subcutaneous connective tissue. The results suggest that one of the effects of the administration of digitoxin in these dosages is to deplete the body store of potassium.

1. Kinsell, L. W., Zillesson, F. O., Smith, A. M, and Palmer, J., *Endocrinology,* 1942, v30, 221.

2. Zwemer, R. L., and Lowenstein, B. E., *Science,* 1940, v91, 75.

3. Aikawa, J. K., and Rhoades, E. L., to be published.

4. Aikawa, J. K., Felts, J. H., Tyor, M. P., and Harrell, G. T., *J. Clin. Invest.,* 1952, v31, 743.

5. Snedecor, G. W., *Statistical Methods,* Iowa State College Press, 1946.

6. Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F., *Am. J. Physiol.,* 1941, v135, 230.

7. Darrow, D. C., and Miller, H. C., *J. Clin. Invest.,* 1942, v21, 601.

8. Aikawa, J. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 105.

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A Practical Method for Removal of Anticomplementary Properties from Human Serum. (22026)

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Methods for the removal of anticomplementary (AC) qualities from sera(1-11) are generally too complex or inconvenient for frequent use in complement-fixation (CF) tests. Since Coons and his colleagues have found that absorption of fluorochrome-labeled antibody with tissue powder reduces or removes nonspecific staining activity(12,13) the effect of tissue powder on the AC properties of human sera was investigated. Sera were freed of AC activity by the relatively simple procedure of treatment with mouse liver powder.

Materials and methods. To make tissue powder, livers from normal adult white mice (Albany strain) were cut into 10- x 10-mm pieces after the removal of fat and connective tissue, and homogenized in a Waring blendor with distilled water (30 ml/100 g). The suspension was centrifuged and the supernatant removed. The sediment was washed 3 times in distilled water, then in 0.85% NaCl solution, and mixed thoroughly with 4 volumes of acetone. The acetone was removed after centrifugation and one volume of acetone was added to the sediment. The acetone was

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evaporated overnight at room temperature in Petri plates. Some samples were further dried in a vacuum desiccator over $CaCl_2$. *Virus and rickettsial antigens.* Virus antigens were prepared in this laboratory. St. Louis encephalitis antigen was a suspension of infected mouse brain(14) inactivated by ultraviolet radiation. The rest were made from tissue or fluids of infected embryonated hens' eggs. Eastern and Western equine encephalomyelitis antigens were extracts of embryo and chorioallantoic membrane suspensions in allantoic fluid also inactivated by ultraviolet radiation. Herpes simplex antigen was an extract of frozen and thawed chorioallantoic membranes. Mumps antigen was dialyzed allantoic fluid. Preparation of the soluble influenza antigens was based on Hoyle's method(15). Methods for production of psittacosis(16) and lymphocytic choriomeningitis(17) antigens have been described. All the virus antigens were stored in the dry state. Rickettsial antigens were Lederle Laboratory products. *Treatment of sera.* Serum was added to the liver powder in the proportion of 200 mg of powder/ml (50 mg/1 ml of 1:4 dilution) and shaken for one hour in a wrist-action machine.‡ The mixture was centrifuged for 30 minutes at 2000-2500 r.p.m. and the supernatant removed from the sedimented liver powder with a fine Pasteur pipette, then inactivated for 30 minutes at 60°C. *Complement-fixation tests.* Treated and untreated sera were appropriately diluted and examined in a quantitative CF test(18) with one, two, and three 50% units of complement in the absence of antigen to determine anticomplementary titer. Serial 2-fold dilutions of serum, beginning with 1:4, were tested with various antigens and three 50% units of complement. Fixation for 18-24 hours at 3°-6°C was followed by incubation for 30 minutes at 37°C with sensitized cells. Antigen controls, complement controls, with one, two, and three 50% units, and a control for sensitized cells were included; and tests with treated and untreated "positive" and "negative" control sera were included with each group of anticomplementary sera examined. Titers were expressed as the number of 50% complement units fixed by undiluted serum. Sera were considered AC if two 50% units of complement induced less than 90% hemolysis in a 1:4 serum dilution in the absence of antigen. The calculated minimum number of 50% complement units fixed by the least AC sera was 5.7 according to this criterion. Many of the sera selected for study were more strongly AC.

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FIG. 1. Effect of liver powder treatment on strongly AC Pool I (20 sera) in CF tests with 12 different antigens. EEE, WEE, SLE = Eastern, Western, and St. Louis encephalitis; LCM = lymphocytic choriomeningitis; RMSF = Rocky Mountain spotted fever.

Results. The objects of the experiments were: To determine the extent to which liver powder removed or inhibited AC activity; to find out if specific antibody titers were altered; and to ascertain whether nonspecific CF properties were introduced by liver powder treatment. Accordingly, both AC sera and sera free of AC activity were examined in CF tests in the absence of antigen, giving the so-called "serum control titer"; in the presence of specific virus or rickettsial antigens; and in the presence of "control" antigens prepared from uninfected tissues or fluids.

Anticomplementary sera. To determine whether liver powder treatment would reduce or remove AC activity, AC sera were pooled and tested prior to and after treatment. Fig. 1 shows the results with one strongly AC pool examined with 12 virus or rickettsial antigens. The untreated serum control fixed thirty-six 50% units of complement, the treated control 6.8, a reduction of 29 units. In all cases, "control" antigens fixed <8 units of complement after treatment. The titers with 6 of the specific antigens were also reduced to <8

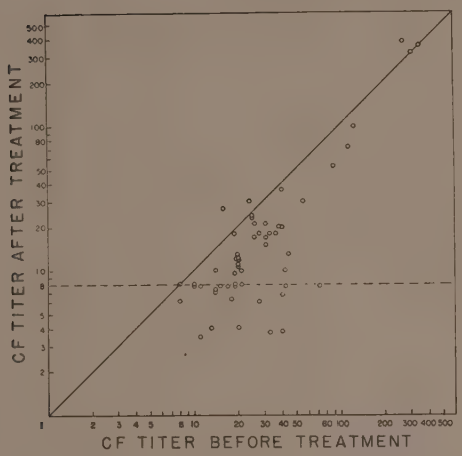

FIG. 2. Effect of treatment on 32 AC sera in 56 tests with 7 different virus or rickettsial antigens.

after treatment, a level considered nonreacting in the test employed. With 4 other antigens, however, namely herpes simplex, mumps, psittacosis, and influenza Type A, some activity remained after liver powder treatment. The reduction corresponded to that obtained in the absence of antigen. It was reasonable to assume, therefore, that the pool contained antibodies to these viruses, and that the procedure did not destroy or denature the antibody. Confirmatory results were obtained with a second moderately AC pool.

In the next experiment, untreated and treated portions of 32 individual AC sera were similarly examined. Liver powder treatment reduced the AC activity of all but one serum, and 22 of the 32 were freed of AC properties, *i.e.*, they fixed less than 5.7 fifty percent units of complement in the absence of antigen. Five of the remaining 10 sera fixed <8 fifty percent units of complement, but 4 markedly AC sera were not affected as decidedly.

To estimate further the effect of treatment on AC activity on these 32 sera, 56 tests were done with different virus or rickettsial antigens and 50 tests with their corresponding uninfected tissue or fluid control antigens. Liver powder treatment decreased the amount of complement fixed with the virus or rickettsial antigen in all but 6 instances (Fig. 2). In 80% of the sera tested, this decrease closely approximated the reduction in AC activity. Table I gives the results obtained with 5 sera from this group tested with various antigens.

Sera free of anticomplementary activity. More conclusive evidence regarding the effect of liver powder treatment on antibody was

TABLE I. Representative Changes in CF Titers of AC Sera Treated with Liver Powder.

Serum No.	Test	Titers: Pre-treatment* A	Titers: Post-treatment* B	Titer differences A–B†
2072	No antigen (AC activity)‡	26	12	14
	With "control" antigen§	20	14	6
	With influenza Type A antigen	36	18	18
1159	No antigen (AC activity)	18	5.3	12.7
	With "control" antigen	20	<8	>12
	With psittacosis antigen	56	30	26
2462	No antigen (AC activity)	16	5.6	10.4
	With Q fever antigen	19	<8	>11
2205	No antigen (AC activity)	19	6.4	12.6
	With "control" antigen	22	9	13
	With influenza Type A antigen	33	18	15
	Idem B	31	17	14
	" C	28	18	10
2091	No antigen (AC activity)	31	22	9
	With "control" antigen	26	21	5
	With mumps antigen	42	30	12

* Pre- or post-treatment with liver powder.
† A–B = Effect of liver powder in decreasing titer.
‡ Determination of AC activity by test in absence of antigen ("serum control").
§ Test in presence of uninfected tissue or fluid antigen corresponding to virus or rickettsial antigen.

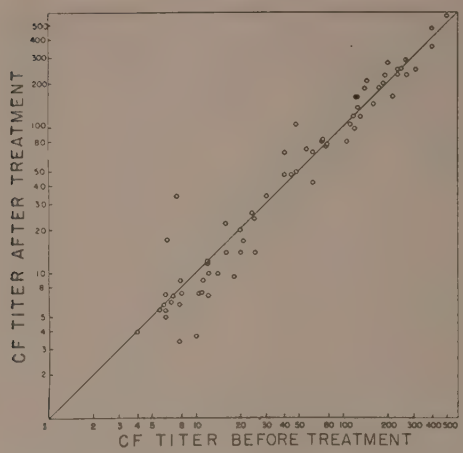

FIG. 3. Effect of treatment on 41 sera free of AC activity in 70 tests with virus or rickettsial antigens.

obtained from studies of sera that had no AC activity. Forty-one sera with titers ranging from <8 to 497 were investigated in 70 tests with virus or rickettsial antigens. Titers of these sera were virtually unaltered by liver powder treatment (Fig. 3). Only 13 (19%) pre- and post-treatment titers differed by more than 25%.

Discussion. Removal of anticomplementary activity without destruction or denaturation of specific complement-fixing antibodies has been a problem from the early days of the CF test. Sachs'(1) method of treatment with diluted HCl depends upon formation of a precipitate that carries down most or all of the AC activity. Maltaner and Maltaner(2,3) modified Sachs' technic. They adjusted the pH of sera to various levels, and concluded that the amount of AC activity removed was proportional to the euglobulin precipitated. For optimum results, it was necessary to adjust the relative concentrations of reagents for each serum. The method was therefore not suitable for large-scale application. Davis *et al.*(4) showed that the AC properties of electrophoretically separated γ-globulin were neutralized by appropriate amounts of β-globulin or albumin. They also investigated high-speed centrifugation as a method for removing AC activity, but concluded that its usefulness is limited. Taran(6) and Maaløe(7), like Davis *et al.*, attempted to neutralize rather than to remove the substances responsible for AC activity. They adopted the direct method of adding complement to replace the amount bound nonspecifically by the AC substances. This method, however, has the disadvantage of decreasing or altering antibody titers; under these conditions, mixtures of complement that are apparently identical with respect to hemolytic potency may differ considerably in their ability to combine with antigen-antibody complex(19). Like the other methods, this technic has the limitation that the appropriate dose of reagent, in this case complement, must be determined for each AC serum.

The method described here is empirical. The effect of liver powder in removing or neutralizing AC properties was studied because tissue powder treatment prevents nonspecific fluorescence(12). The treatment was also highly effective in removing or reducing the AC properties of most of the sera studied, did not reduce specific CF antibody titer in most instances, nor introduce other nonspecific properties that interfere with the CF test to an important degree. Fractionation of the powder would be a promising approach toward explanation of the mechanism of action. Other tissue powders are also being examined for their ability to remove or reduce AC activity.

Preliminary studies suggest that the effect of liver powder on the AC properties of γ-globulin is worth investigation(20). Some results indicate that the method might find application in serologic tests for syphilis with cardiolipin antigen(20). The procedure lends itself well to relatively large-scale work because it is technically simple. In particular, the effective amount of liver powder does not need to be determined separately for each serum specimen. It seems worth while, therefore, to present our data for their possible practical value before an explanation is at hand, in the hope that these observations may prompt further studies of the reaction.

Summary. A simple method for the removal of anticomplementary properties from sera by treatment with acetone-extracted, dried normal mouse liver powder is described.

This material removes nonspecific properties, does not markedly reduce or increase antibody titers with sera giving specific reactions with virus and rickettsial antigens, and does not introduce nonspecific properties in nonreacting specimens.

1. Sachs, H., *Berlin Klin. Wochschr.*, 1921, v58, 1075.

2. Maltaner, F., and Maltaner, E., In N. Y. State Department of Health, Division of Laboratories and Research, *Ann. Rep.*, 1943, p19.

3. ———, *ibid.*, 1945, p14.

4. Davis, B. D., Kabat, E. A., Harris, A., and Moore, D. H., *J. Immunol.*, 1944, v49, 223.

5. Davis, B. D., Moore, D. H., Kabat, E. A., and Harris, A., *ibid.*, 1945, v50, 1.

6. Taran, A., *J. Lab. Clin. Med.*, 1946, v31, 1037.

7. Maaløe, O., *On the Relation Between Alexin and Opsonin. Dissertation,* Munksgaard, Copenhagen, 1946, 186p.

8. Nørgaard, O., *Acta Path. Microbiol. Scand.*, 1950, v27, 811.

9. ———, *ibid.*, 1950, v27, 865.

10. ———, *ibid.*, 1954, v34, 336.

11. ———, *ibid.*, 1955, v36, 71.

12. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

13. Coons, A. H., Leduc, E. H., and Connolly, J. M., *ibid.*, 1955, v102, 49.

14. Casals, J., and Palacios, R., *ibid.*, 1941, v74, 409.

15. Hoyle, L., *Mon. Bull. Minist. Hlth. Lab. Serv.*, 1948, v7, 114.

16. Whitney, E., and Gnesh, G. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 356.

17. Whitney, E., Kraft, L. M., Lawson, W. R., and Gordon, I., *Bact. Proc.*, 1953, p50.

18. Wadsworth, A., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health,* 3d ed., Williams and Wilkins, Baltimore, Md., 1947, pp361-465.

19. Silverstein, A. M., *J. Immunol.*, 1954, v72, 187.

20. Gnesh, G. M., and Rapp, F., unpublished data.

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Inhibition of Development of Chick Embryo Liver by Aminoguanidine. (22027)

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Studies have been made on the nutrition, metabolism, and development of the chick embryo by injection of biologically active substances into the embryonated egg. Profound inhibition of growth with production of developmental abnormalities result from injection of certain substances(1-5), and Karnofsky(6) pointed out the value of this technic as a screening method for specific growth-inhibitors.

By extension of this technic to new classes of chemical compounds, we have observed a severe inhibition of the development of the chick embryo liver by aminoguanidine. This inhibition is rather specific and has no obvious effect on the other organs of the body under the conditions described.

* The authors gratefully acknowledge the technical assistance of Mr. Gene Conway and Mr. Merle Maxwell.

Methods. Aminoguanidine sulfate (AGS)† was dissolved in double distilled water to form

TABLE I. Effect of Aminoguanidine Sulfate on Mortality, Body and Liver Weight of Chick Embryo.*

Quantity injected (mg/egg)	No. of eggs	Embryos survived	Body wt (g)†	Liver wt (mg)†
H_2O	4	4	9.89 ± .89	173 ± 26
2	14	12	8.26 ± .96	139 ± 38
5	8	7	8.58 ± .52	66 ± 39
10	14	8	8.07 ± .69	74 ± 44
15	8	8	9.26 ± .71	65 ± 62
20	14	9	7.11 ± .79	45 ± 36‡
25	8	1	6.75 ± —	— —
40-60	9	0	— —	— —

* Yolk sac injections were made on 4-day-old embryos which were harvested 10 days later.

† Mean value ± avg dev.

‡ Six of the livers at this dosage each weighed less than 25 mg.

† Obtained from Distillation Products Industries, Eastman Organic Chemicals Department.

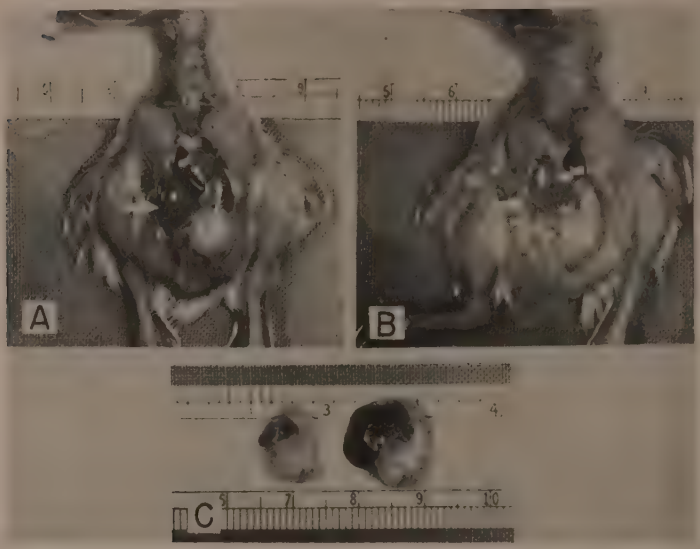

FIG. 1. Gross appearance of 14-day-old chick embryo after injection of 20 mg AGS at the 4th day of incubation. A—Control; B—Treated; C—Comparative size of livers.

a solution of desired maximal concentration with an adjusted pH of 5.6. The solution was sterilized by passage through a Selas filter of 03 porosity. Sterile double distilled water was used for further dilutions. White Leghorn eggs were incubated at 38°C and 80% humidity for 4 days. At the end of this time .4 ml of the solution to be tested was injected into the yolk according to conventional methods. After further incubation (usually until 14 days of age), the embryos were removed and examined. The specimens were preserved in 10% formalin. When histological examination was desired, the embryos of specific organs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

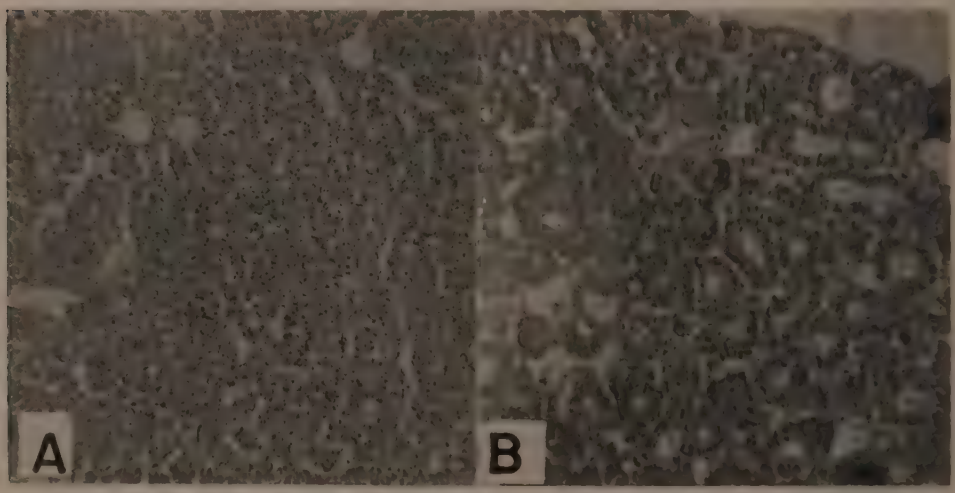

FIG. 2. Section of liver of 14-day-old chick embryo when injected at 4 days (hematoxylin eosin stain, 100 ×). A—Control; B—20 mg of AGS.

TABLE II. Effects of Aminoguanidine Sulfate on Chick Embryo when Administered at Different Embryonic Ages.*

Age at injection (days)	No. of eggs	Embryos survived	Body wt (g)†	Liver wt (mg)†
		H_2O injected		
0- 3	24	17	8.20 ± .66	155 ± 11
4-10	30	26	8.87 ± .74	175 ± 16
		2 mg aminoguanidine sulfate injected		
0	8	5	7.43 ±1.18	157 ± 11
2	8	4	— —	131 ± 25
3	6	4	8.01 ±1.89	75 ± 27
4	14	12	8.26 ± .96	139 ± 38
5	6	6	9.15 ± .96	169 ± 25
6	6	6	7.64 ± .45	161 ± 27
7	6	6	8.44 ± .73	177 ± 17
8	6	5	9.45 ± .60	195 ± 18
9	6	4	9.00 ± .70	180 ± 28
		10 mg aminoguanidine sulfate injected		
2	8	1	— —	5 —
3	6	4	7.96 ± .23	49 ± 13
4	14	8	8.07 ± .69	74 ± 44
5	6	6	8.99 ± .30	56 ± 33
6	6	5	7.70 ± .45	76 ± 19
7	6	2	7.27 ± .20	88 ± 25
8	5	2	9.00 ± .41	166 ± 25
9	6	5	8.00 ± .91	159 ± 9
		20 mg aminoguanidine sulfate injected		
2	6	—	— —	— —
3	6	2	7.32 ± .03	20 ± 3
4	14	9	7.11 ± .79	45 ± 36‡
5	12	8	7.74 ±1.02	48 ± 20
6	12	9	8.17 ± .57	95 ± 26
7	12	9	7.44 ± .41	97 ± 23
8	6	5	9.01 ± .77	141 ± 24
9	12	8	8.93 ± .79	165 ± 25
10	6	3	10.00 ± .66	182 ± 9

* Each dose was administered in 0.4 ml of solution injected into yolk. Embryos were examined at 14 days of incubation.

† Mean value ± avg dev.

‡ Six of the livers at this dosage each weighed less than 25 mg.

Results. The effect of gradated amounts of AGS on the mortality, body weight and liver mass of the chick embryo examined at 14 days of age is recorded in Table I. The number of embryos surviving 25 mg of AGS was negligible. Although the body weight at lower dosage levels was not greatly depressed, the liver mass underwent a marked reduction. At the 20 mg dosage level, ⅔ of the livers weighed less than 25 mg each and several of these consisted of only traces of tissue adherent to the duodenum. The relative mass of livers in typical treated and untreated embryos, as it appeared on gross examination, is shown in Fig. 1. The decrease in liver size and the presence of liver lesions were evident as low as 2 mg AGS dosage levels The other organs were relatively normal in gross appearance and histological sections.

The effects of 3 concentrations of AGS administered at embryonic ages varying from 0-10 days were determined (Table II). The survival rate of embryos injected prior to 4 days incubation was quite low; nevertheless, data indicate the liver to be most susceptible to inhibition when treatments are at 2-4 days. As the age of injection increased, the liver mass is decreasingly affected by a given dosage level of AGS. Finally, the liver appears perfectly normal following injection at 9-10 days of age.

In order to observe the progressive effects of AGS on the liver, eggs injected with 20 mg AGS at 4 days were removed at subsequent 48-hour intervals. Histological examination revealed that cord formation is retarded within 96 hours. In the typical liver of a treated 14-day embryo, the cord development was depressed and the parenchymal cells were replaced in large part by connective tissue and tubule-like structures (Fig. 2). Examination of serial sections revealed that these latter structures were actually oval or rounded cysts. Livers of embryos surviving beyond 14 days appeared to resume normal growth.

Discussion. Aminoguanidine has been tested on a variety of tumors and the results were considered negative(7-10). Brochman *et al.* (11) noted significant tumor inhibition by acid hydrazides such as diaminobiuret, isonicotinic hydrazide, and substituted benzoic acid hydrazides when mice bearing Sarcoma 180 were maintained on vit. B_6-deficient diet. This effect was not observed on a complete diet, but an increased restriction of tumor growth resulted when the acid hydrazides were administered in combination with desoxypyridoxine on mice maintained on a B_6-deficient diet. In the present reported studies, when pyridoxal or pyridoxine was administered in 2 mg/egg quantities concurrently with 2-4 mg/egg of AGS, no effect on the liver of the embryo was noted. Guanidine hydrochloride injected into the yolk of a 4-day embryonated egg killed all specimens at the 15

mg/egg level. Smaller quantities produced some minor liver damage but no particular abnormality as was noted in the case of AGS. An inquiry is now being made into the effect of other hydrazides upon the chick embryo. In general, these are lethal in mg doses and produce minor liver damage at lower levels. In no instance have other hydrazides produced the extreme inhibition of the liver as reported here.

Summary. Aminoguanidine sulfate when injected into the yolk of a 4-day embryonated egg produced a severe inhibition of liver development of the chick embryo when the specimens were examined 10 days later. Apparently, the parenchymal cells were the sites of inhibition as the surviving tissue, in addition to being reduced in mass, was composed largely of connective tissue and cystic structures. Other organs and tissues of the specimens appeared to be relatively unaffected. The most effective sub-lethal dose at 4 days incubation was ineffective when administered at 9-10 days of incubation. Other hydrazides possess peculiar effects which bear no resemblance to that of AGS.

1. Karnofsky, D. A., Ridgway, L. P., and Patterson, P. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 447.

2. Karnofsky, D. A., Dagg, C. P., and Lacon, C. R., *Fed. Proc.*, 1955, v14, 439.

3. Ridgway, L. P., and Karnofsky, D. A., *Ann. N. Y. Acad. Sci.*, 1952, v55, 203.

4. Karnofsky, D. A., Ridgway, L. P., and Patterson, P. A., *Endocrinology*, 1951, v48, 586.

5. Landauer, W., *J. Cell. Comp. Physiol.*, 1954, v43, 261.

6. Karnofsky, D. A., *Cancer Research*, 1955, Supp. 3, 83.

7. Field, J. B., Boryezka, A., and Corta, F., *ibid.*, 1955, Supp. 2,3.

8. Skipper, H. E., and Thomson, J. R., *ibid.*, 1955, Supp. 2, 147.

9. Stock, C. C., Clarke, D. A., Phillips, F. S., and Barclay, R. K., *ibid.*, 1955, Supp. 2, 179.

10. Turner, F. C., *ibid.*, 1955, Supp. 2, 339.

11. Brochman, R. W., Schabel, F. M., Jr., Thomson, J. R., and Skipper, H. E., *Fed. Proc.*, 1955, v14, 186.

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Development of Hypertension and Cardiovascular-Renal Lesions During Adrenal Regeneration in the Rat.* (22028)

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During a study of the effect of bilateral adrenalectomy on the production of hypertension and cardiovascular-renal lesions by methylandrostenediol in the rat it was observed that one control animal developed hypertension. Autopsy showed that the right adrenal of this rat had been completely removed but that a large regenerated adrenal cortex was present on the left side attached to the adrenalectomy scar. The possibility came to mind that the hypertension in this animal may have been the result of excessive hormonal secretion by the regenerating adrenal tissue. The following experiment was performed to test this hypothesis.

Methods. Twenty-four female Sprague-Dawley rats averaging 62 g body weight were unilaterally nephrectomized and given a diet of Purina Laboratory chow and 1% sodium chloride solution *ad libitum*. Twelve rats served as controls and 12 were unilaterally adrenalectomized with contra-lateral adrenal enucleation(1). This procedure consists of a small incision in capsule of the gland through which the bulk of glandular tissue is extruded by gentle application of pressure with curved forceps. The capsule and some adherent cells of the zona glomerulosa are

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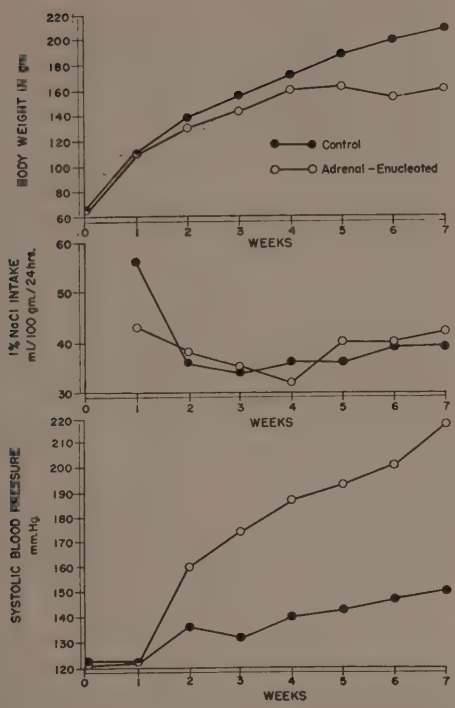

FIG. 1. Body wt, 1% sodium chloride consumption and systolic blood pressure of rats killed at 7 wk.

thus left *in situ*. Body weight, systolic blood pressure(2) and average 24-hour saline consumptions were determined weekly. Six rats in each group were killed at 7 weeks, the remaining 6 in each group at 14 weeks. At each time-interval blood was taken from the abdominal aorta and serum sodium and potassium determined on the Beckman DU flame photometer and chloride estimations made by the method of Schales and Schales(3). Organs were removed, weighed fresh and fixed in either Zenker-formal or 10% formalin. Paraffin sections were stained with hematoxylin and eosin, and frozen sections of adrenals were stained with Sudan IV.

Results. The adrenal-enucleated rats grew slightly more slowly than the controls and at no time did they consume significantly more saline solution than control rats (Fig. 1 and 2). Despite absence of an increased sodium chloride intake the adrenal-enucleated rats developed hypertension beginning from the second to fourth week (Fig. 1, 2). Systolic blood pressure of adrenal-enucleated rats killed at 7 weeks reached a terminal maximum of 217 $\pm$ 9 mm mercury as compared to terminal maximum of 151 $\pm$ 6 mm mercury in control rats. The systolic pressure of the adrenal-enucleated rats killed after 14 weeks levelled off at approximately 200 mm mercury at 9 weeks and had a terminal maximum of 197 $\pm$ 16 mm mercury as compared to 145 $\pm$ 7 mm mercury in control animals. In Tables I and II it can be seen that the serum sodium, potassium and chloride values in the adrenal-enucleated rats did not differ from the controls at either the 7- or 14-week interval.

At 7 weeks there was highly significant enlargement of the heart, kidney and brain in the adrenal-enucleated rats (Table I). The weight of the regenerated adrenal cortex of these rats did not differ from the combined weight of the adrenals in control animals. The

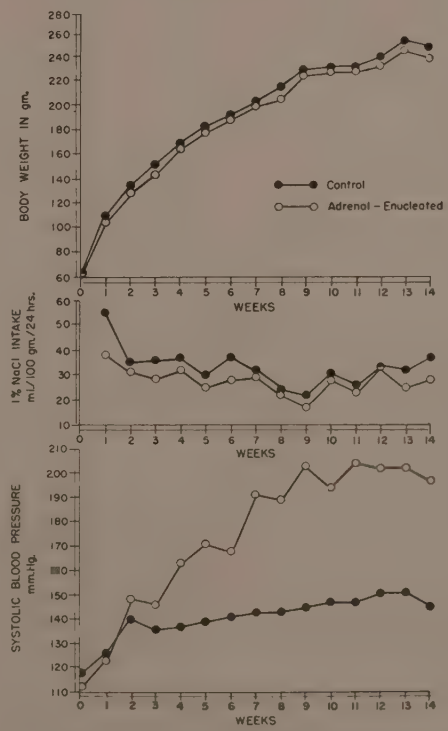

FIG. 2. Body wt, 1% sodium chloride consumption and systolic blood pressure of rats killed at 14 wk.

TABLE I. Organ Weights and Serum Electrolyte Values of Rats Killed at 7 Wk.

	No. of rats	Organ wt (mg/100 g body wt)							Serum electrolytes (mEq/l)		
		Heart	Kidney	Brain	Adrenal(s)	Liver	Thymus	Spleen	Pituitary	Na	K	Cl
Control	6	420 ± 9*	733 ± 36	823 ± 33	31.8 ± 1.0	4120 ± 220	177 ± 17	272 ± 6	4.6 ± .17	143.5 ± 0	4.42 ± .2	106.6 ± 3.4
Adrenal-enucleated	6	588 ± 31	1018 ± 108	1228 ± 34	29.0 ± .6	4987 ± 523	94 ± 22	316 ± 57	4.5 ± .4	145.2 ± 1	4.72 ± .3	110.0 ± 3.2

* Stand. error of the mean.

TABLE II. Organ Weights and Serum Electrolyte Values of Rats Killed at 14 Wk.

	No. of rats	Organ wt (mg/100 g body wt)							Serum electrolytes (mEq/l)		
		Heart	Kidney	Brain	Adrenal(s)	Liver	Thymus	Spleen	Pituitary	Na	K	Cl
Control	6	360 ± 13*	622 ± 44	716 ± 10	26.4 ± 1.2	3093 ± 109	107 ± 16	210 ± 18	4.6 ± .3	145.3 ± .9	3.9 ± .08	108.1 ± 1.8
Adrenal-enucleated	5†	442 ± 29	798 ± 47	753 ± 23	18.2 ± 1.6	3920 ± 249	92 ± 15	221 ± 28	5.6 ± .14	141.2 ±2.0	4.2 ± .3	108.7 ± .8

* Stand. error of the mean.
† One rat died before termination of the experiment.

thymus was significantly atrophic but nevertheless present in all animals bearing regenerating adrenals. No lesions were observed in control rats but in the adrenal-enucleated animals all 6 showed renal lesions, 5 showed cardiac lesions and 5 had periarteritis nodosa in either the pancreas or mesentery or both. Cerebral changes were observed in 5 adrenal-enucleated rats and consisted of focal cerebral hemorrhages and neuronal degeneration, edema and cyst formation with bulging of the cranial vault. Microscopically the renal and cardiac lesions and periarteritis nodosa were identical to those regularly produced by overdosage of desoxycorticosterone acetate(4, 5,6), and, except for the presence of periarteritis nodosa, are remarkably similar to the lesions of malignant hypertension in the human being.

In animals sacrificed after 14 weeks, the weights of kidney and heart of the adrenal-enucleated rats were significantly greater than in the controls (Table II) but less markedly than at 7 weeks. There was no cerebral enlargement or edema in the rats bearing regenerated adrenals. The weight of the regenerated adrenal gland was now significantly less than the combined weight of the 2 adrenals of the control animals, and the weight of the thymus of adrenal-enucleated rats was the same as that of controls. Although some control rats had developed scattered glomerular lesions similar to those in the enucleated rats, the lesions were more widespread and more severe in the latter animals. In general, however, the lesions in the adrenal-enucleated animals were less severe at 14 weeks than at 7 weeks. For example, mesenteric and pancreatic periarteritis nodosa was found in only one rat and no cerebral lesions were detected.

Discussion. Considerable evidence has been presented in support of the hypothesis (4) that derailment of the General-Adaptation-Syndrome involving hypersecretion of the adrenal cortex may lead to malignant hypertension and cardiovascular-renal lesions in the human being. This theory has been largely based on experiments in rats in which adrenocortical hyperfunction has been produced by exposure of the organism to nonspecific stress(7), the administration of either

anterior hypophyseal trophic substances(8), or corticoid hormones(9). Because the factor of non-specific stress has not seemed to play a prominent part in human hypertension and cardiovascular-renal disease many investigators have remained unconvinced of the importance of adrenocortical hyperfunction in their pathogenesis(10,11). Without assuming that there must be such a pathogenetic relationship, as outlined above, it is considered of some importance that the present experiment has demonstrated that hypertension and cardiovascular-renal lesions can be produced apparently as a result of hyperfunction of the adrenal cortex under conditions in which exogenous non-specific stress seems to play no part. The stimulus to both regeneration and to hyperfunction appears to lie entirely within the organism, and on the basis of extensive studies by Ingle(12) is assumed to be the lowered corticoid blood levels produced by the hemi-adrenalectomy and contra-lateral adrenal enucleation which in turn leads to increased secretion of ACTH by the anterior hypophysis.

Some thought concerning the type of secretion of the adrenal cortex under these conditions may be pertinent. Ingle has shown(12) that continuous administration of ACTH to adrenal-enucleated rats can cause such profound weight loss that the animal is brought to the point of death, presumably as a result of the greatly increased secretion of gluco-corticoids. Furthermore cortisone has been shown to produce hypertension and cardio-vascular-renal changes similar to those observed in the present experiment(13,14). However, this steroid produces increased consumption of sodium chloride drinking solution, profound thymic and splenic atrophy and does not cause the production of periarteritis nodosa but rather promotes the healing or prevents the development of such a lesion(13, 14). In this experiment no polydipsia occurred, thymic atrophy was never profound and present only at the 7-week interval, splenic atrophy did not occur and periarteritis was produced. Of further interest is the total absence of any significant changes in the serum electrolyte pattern.

All these observations suggest that the regenerating adrenal cortices were not secreting increased quantities of gluco-corticoids alone but rather that there was increased secretion of some mineralo-corticoid either alone or together with gluco-corticoids. Such a possibility is entirely compatible with the observations of Singer and Stack-Dunne(15), who demonstrated that the rat adrenal can respond to ACTH by liberation of both corticosterone and aldosterone into the adrenal vein. Furthermore it must be borne in mind that enucleation removes the zones which are presumed to secrete gluco-corticoids but leaves the zona glomerulosa which is considered to be the source of mineralo-corticoids, a situation which in itself might lead to imbalance of corticoid secretion. Regardless of how adrenocortical function may be altered in the present experiment the method makes possible future studies of the steroid secretory pattern of the adrenal cortex under conditions of increased function in the absence of superimposed exogenous stress and in which hypertension and vascular disease are a natural consequence.

Summary and conclusions. This report presents a simple technic for the production of a new type of experimental hormonal hypertension with cardiovascular-renal lesions in rats sensitized by unilateral nephrectomy and salt administration. It demonstrates that such hypertension and cardiovascular-renal lesions can be produced without polydipsia and excessive sodium consumption and without alteration in the serum levels of sodium, potassium or chloride. The results suggest that regenerating adrenal cortical tissue secretes increased quantities of cortical hormones and lend strong support to the hypothesis that increased adrenal cortical function is of fundamental importance in the pathogenesis of hypertension and cardiovascular-renal disease.

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1. Ingle, D. J., and Higgins, G. M., *Am. J. Med. Sci.*, 1938, v196, 232.

2. Friedman, M., and Freed, S. C., PROC. SOC.

EXP. BIOL. AND MED., 1949, v70, 670.

3. Schales, O., and Schales, S., *J. Biol. Chem.*, 1941, v140, 879

4. Selye, H., *Stress,* Acta Inc., Montreal, Canada, 1950.

5. Masson, G. M. C., Hazard, J. B., Corcoran, A. C., and Page, I. H., *A. M. A. Arch. Path.*, 1950, v49, 641.

6. Skelton, F. R., *ibid.*, 1955, v60, 190.

7. Selye, H., *Rev. Canad. de biol.*, 1943, v2, 501.

8. Selye, H., Stone, Helen, Nielson, K., and Leblond, C. P., *Canad. M.A.J.*, 1945, v52, 571.

9. Selye, H., Hall, C. E., and Rowley, E. M., *ibid.*, 1943, v49, 88.

10. Rosenberg, C. A., Woodbury, D. M., and Sayers, G., *J. Clin. Endocrinol.*, 1952, v12, 666.

11. Ingle, D. J., and Baker, B. L., *Recent Progress in Hormone Research,* Academic Press, New York, 1953, v8, 143.

12. Ingle, D. J., *Pituitary-Adrenal Function* (A Symposium), Am. Assn. Adv. Sci., Washington, D.C., 1950.

13. Selye, H., *Second Annual Report on Stress,* Acta Inc., Montreal, Canada, 1952.

14. Skelton, F. R., unpublished observations.

15. Singer, Bertha and Stack-Dunne, M. P., *J. Endocrinol.*, 1955, v12, 130.

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Gonadotrophic Hormone Excretion of the Pregnant Monkey (*Macaca mulatta*).* (22029)

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The object of this study was to re-examine the reports that gonadotrophic hormone is excreted by the pregnant monkey for an exceedingly limited period of time. To determine simultaneously the type of gonadotrophin excreted by the monkey, the hypophysectomized rat was used to assay the urine. The first precise evidence for excretion of gonadotrophin by the pregnant monkey was furnished by Hamlett(1), who reported a positive Friedman reaction from urine collected between the 19th and 26th days. No urine collected after the 26th day of pregnancy gave a positive test. Delfs(2) found that gonadotrophic hormone could be detected (by the rat uterine-weight test) in the blood serum of pregnant monkeys as early as the 14th day of pregnancy; the amount reached a peak by the 20th to 25th day and the hormone disappeared by the 30th day. In both of these studies the time of gestation was accurately known from cycle and breeding dates. It appears that monkeys resemble man in that gonadotrophin is present in blood and urine during pregnancy, the hormone appearing in blood and urine soon after the establishment of the placenta; however, in the macaque, the hormone appears to persist for only a short period, whereas in apes (3-5) and man it is present to the end of pregnancy.

Methods. Female rhesus monkeys, *Macaca mulatta,* of the Yale obstetrics colony were placed with males at noon of the 11th day of the menstrual cycle. Each day thereafter a vaginal lavage was examined for the presence of sperm. If sperm were recorded on the 12th day and this was followed by pregnancy, day 12 was considered to be the day of conception. Urine was collected at representative periods from females in which the span of gestation was thus accurately known. Animals were placed in the metabolism cage at noon, for a 24-hour period, and the date of the 1st day identified that urine specimen. The urine drained through a paper filter and was collected under toluol. It was frozen immediately on termination of the collection period. To concentrate for injection, the urine was filtered, acidified to pH 5 and precipitated in 3 times its volume of ethanol. The precipitate was

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TABLE I. Occurrence of Chorionic Gonadotrophin in the Urine of Seven Pregnant Macaques. As demonstrated by ovarian histology in hypophysectomized female rats.

Days	Mm 485 1954	Mm 485 1955	Mm 639	Mm 736	Mm 416	Mm 666	Mm 637	Mm 615
22	+		+					
23								
24	(toxic)		+*					
25				—				
26			+					
27	(toxic)							
28			—		+			
29								
30			+	—				
31	—	+*			—			
32		+*	—	—				
33	—	+*			—			
34		+*	—					
35					—			
36	—		—					
37								
38			—					
39					—			
40			—					
42	—		—					
44			—					
46						—		
48			—		—			
49	—							
51						—		
56	—				—			
62							—	
63					—			
71					—			
73							—	
76	—							
88						—		
120								—
177								—
173-179							—	

* Also gave a positive A-Z test in one normal rat (large follicles and corpora lutea) at a total dose equivalent to ¼ day's output.

washed with 95% ethanol, dissolved in water and dialyzed. Any insoluble material present after dialysis for 24 hours was removed by centrifugation and the clear supernatant was lyophilized. The powder thus obtained from the 24-hour urine sample was extracted by 16 cc water, and 1 cc aliquots were injected, subcutaneously, daily for 4 days, into each of 3 hypophysectomized female rats (26-28 days of age at operation, 8 days postoperative). Each rat, therefore, received the equivalent of ¼ day's urine output. Autopsy was performed 96 hours after onset of the injections. Before use in assay the rats were screened for completeness of hypophysectomy, and at autopsy the success of the operation was further checked so that no data from incompletely hypophysectomized rats were included. The ovaries and uteri were examined carefully under a binocular and weighed. The ovaries were subjected to microscopic analysis for study of the type of gonadotrophin present. Thyroids and adrenals were also examined microscopically for evidence of the presence of thyroid and adrenal stimulating substances in the urine. The width of the uncalcified portion of the proximal epiphyseal cartilage of the tibia was measured for evidence of growth-promoting substance.

Results. (Table I). The chief gonadotrophic effect of the pregnant monkey urine, as judged by the response of ovaries of hypophysectomized rats, was stimulation of the interstitial tissue. Urine collected between days 22 to 34 caused repair or even hypertrophy of interstitial cells when injected in the equiva-

lent of one-fourth of a day's output. From the 35th day to the 179th day, the latest day tested, the concentrates of urine had no gonadotrophic action at the dose tested.

Follicle stimulation was not detected unequivocally in the urinary equivalents injected. An increase in the number of small follicles and some slight increase in follicle size above control level were observed on injection of urines collected between days 22 and 34. The degree of stimulation was characteristic of that seen on injection of minimal, or immediately subminimal doses of pituitary follicle stimulating hormone. There was, however, no evidence of secretion of estrogenic substances judged either from uterine weight or from vaginal reaction. Delfs(2) likewise found that the interstitial tissue of the hypophysectomized rat's ovary was the only tissue responding to the serum from monkeys in early pregnancy. It will be remembered that this selective type of ovarian reaction in the hypophysectomized rat is also typical of human chorionic gonadotrophin.

The response of the ovary of the intact rat to monkey urine gonadotrophin was compared with the well known response to human chorionic gonadotrophin by injecting normal immature rats with the urinary concentrates. One-fourth daily output of urine collected between days 24 and 34 caused the development of a few large follicles and a limited number of corpora lutea, with ovulation. The ovarian reaction in normal immature rats was, therefore, comparable to that obtained after injection of low doses of human pregnancy urine.

It should be noted that ¼ daily urinary output represented approximately 100 to 125 cc of urine. In the usual Aschheim-Zondek test of human pregnancy urine a total of only 4 cc is injected. In assay of human pregnancy urine in hypophysectomized female rats, Lyon *et al.*(6) reported follicular stimulation from ⅕ daily output of urine, or the equivalent of 200 to 300 cc per rat.

A low grade stimulation of the thyroid of hypophysectomized rats, such as is noted on injection of minimal doses of pituitary thyrotrophic hormone, was occasionally observed on injection of urines collected between the 22nd and the 177th day of pregnancy. (The thyroid epithelium was slightly taller, the nuclei were more vesicular, the cytoplasm slightly more abundant, and the colloid somewhat vacuolated and lighter-staining.) These results were therefore suggestive that small amounts of thyrotrophic hormone were present in the pregnancy urine. There was no consistent evidence for adrenocorticotrophic substance, judged either from adrenal weights or histology. Likewise, the uncalcified portion of the proximal epiphyseal cartilage of the tibia of the hypophysectomized rats was in no instance wider than that of uninjected controls, so there was no evidence of excretion of growth-promoting substance.

Summary. The urinary excretion of gonadotrophin by pregnant *Macaca mulatta* was assayed at intervals from day 22 to 179 of gestation. Hypophysectomized immature female rats were injected with concentrates equivalent to ¼ day's output of urine. Only urine excreted between days 22 and 34 of pregnancy had a gonadotrophic action, and the action was restricted to repair of the interstitial tissue. The gonadotrophic stimulus observed in normal immature rats was comparable to that from low doses of human chorionic gonadotrophin during this extremely limited period of pregnancy.

1. Hamlett, G. W. D., *Am. J. Physiol.*, 1937, v118, 664.

2. Delfs, E., *Anat. Record*, 1941, v79 (suppl.), 17.

3. Schultz, A. H., and Snyder, F. F., *Bull. Johns Hopkins Hosp.*, 1935, v57, 193.

4. Zondek, B., *Die Hormone des Ovariums und des Hypophysenvordenlappens*, 1931, Berlin, Springer.

5. Zuckerman, S., *Am. J. Physiol.*, 1935, v110, 597.

6. Lyon, R. A., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1953, v53, 674.

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Comparative Effects of Dietary Sucrose and Cornstarch on Tryptophan Requirement of the Rat.* (22030)

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Available data indicate that the growth increment of rats fed low protein rations is dependent to a considerable degree on the source of dietary carbohydrate. Krehl *et al.*(1), Henderson *et al.*(2), Harper and Katayama (3) and others(4-6) observed that dextrin or cornstarch support better growth of rats fed low casein rations than does sucrose. Similar results have been reported by Womack *et al.* (7) for rats fed a low level of an amino acid mixture. However, the differences in growth on these diets were eliminated when the casein content of rations or the level of the amino acid mixture was increased. In the present communication data are presented indicating that the source of dietary carbohydrate may also affect growth increment of rats fed a diet deficient in a single amino acid.

Procedure. Two basal rations were employed, diets A and B. Diet A was a tryptophan-free ration of the following composition in percent: sucrose, 60.8; acid hydrolyzed casein,† 24; cottonseed oil (Wesson), 10; salt mixture,‡ 5; and l-cystine, 0.2. To each kg were added the following crystalline vitamins: thiamine hydrochloride, 40 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 100 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; choline chloride, 2 g; vit. A, 5000 U.S.P. units; vit. D_2, 500 U.S.P. units; and alpha tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Diet B was similar in composition but contained cornstarch in place of sucrose as the dietary carbohydrate. In addition to the basal rations the following diets were also employed, consisting of basal rations A and B plus each of the following supplements: (a) 600 mg dl-tryptophan/kg diet (b) 1.8 g dl-tryptophan/kg diet and (c) 5.4 g dl-tryptophan/kg diet. These supplements were incorporated in the diet in place of an equal amount of hydrolysate and corresponded to 0.25%, 0.75% and 2.25% respectively of the hydrolysate content of the basal ration. In addition to above diets, experiments were also conducted with diets which were identical to basal rations A and B except that the acid hydrolysate was replaced by intact casein.§ Two hundred albino female rats of the University of S. California strain, were selected at 21 to 24 days of age and average body weight of 42 g (range 36 to 50 g). Animals were placed in metal cages with raised screen bottoms and fed above diets *ad lib.* (20 rats/group). Diets were prepared weekly and stored under refrigeration. Rats were fed daily, and all food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for 18 weeks or until death, whichever occurred sooner. Of the surviving animals, 12 in each group were autopsied and gonadal and uterine weights determined. Ovaries were fixed in Bouin's solution, and sections were prepared and stained with hematoxylin and eosin.

Results are summarized in Table I. Findings indicate that the weight increment of immature rats fed a tryptophan-free diet supplemented with 600 mg dl-tryptophan per kg diet was more than twice as great on

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† The authors are indebted to Dr. Harry Wax of the Stuart Co., Burbank, Calif., for the acid hydrolyzed casein used in this study.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

§ Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

TABLE I. Effects of Dietary Sucrose and Cornstarch on Body, Ovarian and Uterine Weights of Rats Fed a Tryptophan Deficient Ration Supplemented with Graded Levels of dl-tryptophan (20 Animals per Group).

Dietary group	Tryptophan supplement, g/kg of diet	Increment in body wt after 18 wk,* g	Ovarian wt, mg	Uterine wt, mg
S†	.6	57 ± 5.1	18	66
C	.6	128 ± 5.2	45	348
S	1.8	152 ± 4.1	42	300
C	1.8	148 ± 6.0	51	380
S	5.4	146 ± 5.3	49	263
C	5.4	151 ± 3.7	47	298
S	Intact casein	199 ± 4.9	58	398
C	" "	200 ± 6.0	64	483

* Including stand. error of the mean.
† S = Sucrose; C = Cornstarch.

the cornstarch-containing ration than on diet containing sucrose as the source of carbohydrate. Average weight increment of rats at this level of tryptophan supplementation after 18 weeks of feeding was 128 ± 5.2‖ g in the corn starch series and 57 ± 5.1 g for animals fed the sucrose-containing diet. At higher levels of tryptophan supplementation (1.8 g and 5.4 g dl-tryptophan/kg diet), no significant difference in weight increment was observed between rats on cornstarch or sucrose-containing rations. Similarly, no significant difference in weight increment occurred between these two dietary groups when intact casein was fed in place of the hydrolysate. Animals fed unsupplemented tryptophan-free rations A and B lost weight from the start of feeding and died within 6 weeks of start of experiment. A marked difference was noted, however, between rats in the two groups. On the sucrose-containing diet animals developed an unthrifty appearance with ruffled fur and varying degrees of alopecia. On the cornstarch-containing ration, although weight loss of rats was the same as in the sucrose group, the fur remained smooth and sleek. After 28 days of feeding only 1 out of 20 rats fed the basal ration A was still alive in contrast to 16 out of 20 rats on ration B. The average survival time for rats on tryptophan-free diets was 24 ± 0.6 days (range 20 to 29 days) on ration A and 33 ± 1.3 days (range 23 to 41 days) on ration B.

At autopsy significant differences in ovarian and uterine weights were noted between cornstarch and sucrose series in rats fed a tryptophan-free ration supplemented with 600 ml dl-tryptophan/kg diet. On the sucrose-containing diet the ovaries and uteri appeared immature both in weight and microscopic appearance. These findings might have been anticipated since the corresponding organs of rats of similar weight which had been fed a natural food stock ration were also immature. In contrast rats in the cornstarch series at the same level of tryptophan supplementation had well developed ovaries and uteri¶ in addition to increased body weight. At higher levels of tryptophan supplementation (1.8 g and 5.4 g dl-tryptophan/kg diet) and on intact casein rations, the ovaries and uteri were well developed in both the sucrose and cornstarch-containing series.

The weight increment of rats fed dl-tryptophan at levels of 1.8 g and 5.4 g per kg of diet was significantly greater both in the cornstarch and sucrose series than that of animals receiving the lower level of supplement (600 mg dl-tryptophan per kg of diet) but was still approximately one-third lower than that of rats fed the intact casein. Since the 1.8 g level of supplementation was as effective as the higher level of supplement in promoting the weight increment of rats fed the hydrolysate-containing rations, and since the weight increment of rats fed these supplements both in the cornstarch and sucrose series was significantly less than that of animals fed the intact casein, it would appear that the acid hydrolysate employed in the present experiment may have been deficient not only in tryptophan but in some other factor or factors present in the intact casein.

‖ Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

¶ Subsequent data indicate that ovaries of rats in this group were normal functionally as well as in histologic appearance. After the 18th week of feeding, 6 animals in this series were bred to males of proven fertility. All 6 gave birth to living young which appeared normal in all respects except for size (young averaged 3.8 g at time of birth).

Discussion. Findings suggest that the source of dietary carbohydrate may profoundly affect the biological value of a protein. Rats fed a tryptophan deficient ration supplemented with a suboptimal level of dl-tryptophan (600 mg/kg of diet) grew poorly when sucrose was the dietary carbohydrate but showed a significant increment in body weight over that observed on the sucrose-containing diet when cornstarch replaced sucrose as the source of dietary carbohydrate. At higher levels of tryptophan supplementation (1.8 g or 5.4 g dl-tryptophan/kg of diet) no significant difference in weight increment occurred between rats fed cornstarch or sucrose-containing rations. Similar findings were obtained in respect to ovarian and uterine weights. Ovaries and uteri appeared infantile in rats fed a tryptophan-deficient ration supplemented with suboptimal levels of dl-tryptophan when sucrose was the dietary carbohydrate but appeared normal in size and appearance either when cornstarch replaced sucrose as the source of dietary carbohydrate or when the tryptophan content of the sucrose-containing ration was increased.

No data are available to account for the beneficial effects of cornstarch over sucrose for rats fed suboptimal amounts of tryptophan under conditions of the present experiment.** It has been suggested that essential amino acids may be synthesized by the intestinal flora of rats fed cornstarch(4) and it is possible that sufficient amounts of tryptophan may be available from this source to account for the observed results. The converse explanation—that the intestinal flora of rats fed sucrose use greater quantities of tryptophan than the intestinal flora of animals fed cornstarch—would appear, however, to be equally tenable(3). Another hypothesis is that the rate of digestion may be slower when rations containing cornstarch in place of sucrose are fed and that this may permit a more efficient utilization of tryptophan(5). Regardless of the final explanation of the observations reported above, however, findings clearly indicate that the source of dietary carbohydrate may, at least under certain experimental conditions, significantly modify the tryptophan requirement of the rat.

** In view of the fact that some starches contain small amounts of protein, the possibility was considered that sufficient amounts of tryptophan were derived from this source to account for the observed results. Nessler nitrogen determinations of the cornstarch employed in this experiment, however, revealed no detectable N.

Summary. Immature female rats were fed tryptophan-free rations supplemented with graded levels of dl-tryptophan and containing either sucrose or cornstarch as the source of dietary carbohydrate. Rats fed dl-tryptophan at a level of 600 mg/kg of diet grew poorly when sucrose was the dietary carbohydrate but showed a significant increment in body weight over that observed on the sucrose-containing diet when cornstarch replaced sucrose as the source of dietary carbohydrate. At higher levels of tryptophan supplementation (1.8 g or 5.4 g dl-tryptophan/kg of diet) no significant difference in weight increment occurred between rats fed cornstarch or sucrose-containing rations.

1. Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A., *J. Nutrition,* 1946, v31, 85.

2. Henderson, L. M., Deodhar, T., Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.,* 1947, v170, 261.

3. Harper, A. E., and Katayama, M. C., *J. Nutrition,* 1953, v49, 261.

4. Hall, W. K., and Sydenstricker, V. P., *Arch. Biochem.,* 1946, v12, 147.

5. Lyman, R. L., and Elvehjem, C. A., *J. Nutrition,* 1951, v45, 101.

6. Hankes, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A., *J. Biol. Chem.,* 1948, v174, 873.

7. Womack, M., Marshall, M. W., and Parks, A. B., *J. Nutrition,* 1953, v51, 117.

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Interrelationships of Methionine and Vitamin B_6 in Human Subjects.* (22031)

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(Introduced by C. A. Elvehjem.)

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That vit. B_6 is involved with the metabolism of methionine has been shown by various investigators. Miller and Baumann(1) and Cerecedo and Foy(2) found that high protein diets increased the vit. B_6 requirement. Later work indicated that the adverse effects of high protein diets are due in part to the methionine content of the protein. Methionine in excess of the normal requirement resulted in depressed growth(3-5), an effect particularly evident when the diet was low in vit. B_6(6,7). Linkswiler *et al.*(8) added only 0.4% methionine to an assay diet containing 18% casein and observed that the symptoms of deficiency of the vitamin were aggravated sufficiently that the rat assay could be conducted without a preliminary depletion period. It was observed by DeBey *et al.*(9) that while vit. B_6 counteracted the effects of moderate amounts of methionine, with 2.5% methionine, high levels of the vitamin failed to restore growth. The role of B_6 and methionine in the formation of N^1-methylnicotinamide (NMeN) from nicotinamide has been suggested by other workers. Handler and Dann(10) suggested that nicotinamide is methylated *in vivo* by methionine and excreted in the urine as NMeN. In studies on the urinary excretion of NMeN, Rosen *et al.*(11) noted that B_6-deficient rats did not excrete NMeN. When pyridoxine was administered to these animals, there was a very gradual reappearance of NMeN. Perlzweig *et al.*(12) demonstrated that NMeN is formed *in vitro* by rat liver slices from nicotinamide but not from nicotinic acid. Ellinger (13) confirmed these results and showed that the particular enzyme involved was specific for nicotinamide but not for nicotinic acid. These workers(12,13) observed that the extent of transformation was increased by the addition of methionine to the system. No report on the study of the effect of both pyridoxine and methionine in NMeN formation in man has been found in the literature.

The present investigation was designed to study the effects of pyridoxine and methionine on NMeN formation in man and the effect of a high intake of methionine on the urinary excretion of B_6 and its metabolites by subjects on a diet low in vit. B_6.

Methods. Four normal adult women participated in each experiment, 3 of them taking part in both experiments. This study consisted of 2 experiments of 21 days each, with 3 months intervening between them. Each experiment was divided as follows: a 9-day period during which the subjects received only the basal diet followed by two 6-day periods in one of which the basal diet was supplemented by 8 g of dl-methionine and in the other by 8 g dl-methionine and 10 mg of pyridoxine hydrochloride. The methionine was

TABLE IA. Basal Diet Low in Vit B_6.

Food	Wt, g	Vit B_6 (analytical value), mg	Methionine (calculated value), mg
Orange juice, frozen	100	.021	3.0
Peaches, canned	100	.018	2.0
Apple sauce	100	.001	3.0
Lettuce	10	.003	
Carrots, raw	30	.008	
Celery	5	.017	
Onion	5	.004	
Cream of wheat, dry	25	.024	58.0
Rice, uncooked	50	.045	53.8
Cottage cheese, dry	30		142.5
Gelatin	5		36.0
Biscuits*		.026	
Minerals*			
Vitamin supplements*			
Totals		.167	528.7

* Composition given in Table IB.

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TABLE IB. Basal Diet—Continued.

	g
Biscuits:	
Cake flour	100
Baking powder	8
Crisco	34
Vitamin-free casein	40
Water	70
Salt mixture:	
$CaCO_3$	1.2
$KHCO_3$	1.88
$Mg\ SO_4\ 7H_2O$	.52
$Fe_2\ (C_6H_5O_7)_3\ 3H_2O$	.14
$Mn\ SO_4 \cdot 4H_2O$	Trace
$Cu\ SO_4 \cdot 5H_2O$	"
KI	"
Vitamins:	
Thiamine hydrochloride	1.2
Riboflavin	1.5
Niacin or nicotinamide	10.0

divided into 3 portions, one portion being taken with each meal. Pyridoxine was taken with breakfast during the last period. A supplement of 10 mg niacin was given during the first experiment and 10 mg of nicotinamide, during the second. The *basal diet* for the entire experiment is given in Table I. This diet was found by analysis to furnish 0.167 mg of vit. B_6 per day and 58 g of protein. Forty grams of the protein came from "vitamin-free" casein which was incorporated in biscuits. By computation, the diet was found to contain 428 mg of methionine and 2.69 mg of niacin. Cookies consisting of butter, sugar and cornstarch furnished additional calories to meet the energy requirement of each individual. The intake for each subject remained constant throughout the experiment. After a 3-day preliminary period in each experiment, 24-hour urine collections were made and preserved with toluene and 5 ml of glacial acetic acid. These were pooled into 3-day periods and samples were stored in a refrigerator for analyses. The B_6 content of the food and the urine was determined using the method of Atkin *et al.*(14). The 4-pyridoxic acid in the urine was determined by the method of Huff and Perlzweig as modified by Sarett(15); NMeN was determined according to the acetone-fluorometric method of Huff and Perlzweig(16).

Results. Effect of pyridoxine and methionine on N^1-methylnicotinamide excretion. The data on the urinary excretion of NMeN are presented in Table II. The fourth subject differed in the 2 studies but the values are shown consecutively for comparison. From these data it may be concluded that the methionine supplement and the later addition of pyridoxine hydrochloride to the diet low in B_6 had no effect on the urinary excretion of NMeN when the diet was supplemented with nicotinic acid. This was observed in all 4 subjects. During the low B_6 period, when nicotinamide was the supplement, the excretion of NMeN was approximately the same as when nicotinic acid was taken. The addition of methionine alone had little effect on the excretion. However, when pyridoxine was added in the presence of methionine the NMeN excretion rose markedly for each subject. For 3 subjects the excretion during this period was equal to more than 100% of the nicotinamide supplement and for the fourth subject, it was equal to approximately 65% of the supplement.

If nicotinic acid were readily converted to nicotinamide in the body, one would expect the same effect from both supplements. The increased excretion of NMeN observed only when the supplement was in the form of nicotinamide but not when it was nicotinic acid demonstrates the difficulty in the formation of NMeN from nicotinic acid in man. These results agree with the findings of Perlzweig and his coworkers(12) and Ellinger(13), using rat liver preparations.

The role of B_6 in NMeN formation is shown

TABLE II. Average Daily Urinary Excretion of N^1-methylnicotinamide.

Subject	Basal diet, mg	Basal + 8 g dl-methionine, mg	Basal + 8 g dl-methionine + 10 mg pyridoxine HCl, mg
Period I—Nicotinic acid supplement			
RZ	5.20	4.24	4.02
LM	4.76	5.10	5.10
KC	3.18	4.42	4.32
RB	3.56	5.57	4.74
Period II—Nicotinamide supplement			
RZ	8.95	7.20	11.95
LM	3.75	4.50	12.75
KC	5.85	6.15	14.20
AW	5.40	5.40	6.85

TABLE III. Average Daily Urinary Excretion of Vitamin B_6 and 4-Pyridoxic Acid by Human Subjects on a Diet Low in B_6 and Supplemented with dl-Methionine and with dl-Methionine and Pyridoxine HCl.

	Subject RZ		Subject LM		Subject KC		Subject RB	
	Vit B_6	4-P.A.	Vit B_6	4-P.A.	Vit B_6	4-P.A.	Vit B_6	4-P.A.
Period 1	mg							
Basal diet*	.06	2.48	.04	1.51	.02	2.74	.04	2.72
Basal diet + 8 g dl-methionine	.04	2.69	.03	2.35	.06	2.40	.04	3.46
Basal diet + 8 g dl-methionine + 10 mg pyridoxine hydrochloride	.47	8.10	.39	6.82	.59	5.68	.60	8.17
Period 2							Subject AW	
Basal diet	.06	2.38	.05	1.98	.06	2.57	.06	2.39
Basal diet + 8 g dl-methionine	.06	2.15	.06	2.63	.06	2.60	.07	3.11
Basal diet + 8 g dl-methionine + 10 mg pyridoxine hydrochloride	.53	8.37	.47	6.99	.63	6.62	.63	6.51

* Basal diet furnished 0.16 mg B_6.

by the increased excretion by human subjects of NMeN in the presence of both pyridoxine and methionine but not of methionine alone. A more complete picture of the role of B_6 in the methylation of nicotinamide might have been obtained had there been included an additional period in which pyridoxine without methionine was the supplement.

Urinary excretion of B_6 and metabolites. Data on the urinary excretion of B_6 and 4-pyridoxic acid are presented in Table III. The 4-pyridoxic acid values have been converted to pyridoxine by using the factor 0.92 based on the molecular weights of the 2 compounds. From these data, it appears that a high intake of methionine had no effect on the urinary excretion of vit. B_6 and 4-pyridoxic acid. Similar results were obtained in both experiments. De Bey(9) reported that dietary methionine had no effect on the concentration of vit. B_6 in the blood or liver of rats.

It may be noted from Table III that the 4-pyridoxic acid excretion exceeded the intake of vit. B_6 during the first 2 periods of the study. The amount of B_6 in the diet was 0.167 mg per day, whereas the excretion of 4-pyridoxic acid and B_6 ranged from 1.55 to 3.55 mg per day. Linkswiler *et al.*(17) reported a similar elimination of B_6 and metabolites in excess of intakes of 0.78 mg and 2.78 mg per day.

The fact that the diet used in the current study was almost a synthetic one and excluded foods reported by Sarett(15) to contribute interfering fluorescent substances in the urine, makes it likely that the fluorescent substance measured was actually 4-pyridoxic acid. The 10-mg supplement given the subjects during the last period of the study accounts for the high excretion of B_6 and 4-pyridoxic acid during this period.

Summary. 1. A study of the interrelationship of methionine and pyridoxine was made in adult human subjects maintained on a semi-synthetic diet, low in vit. B_6. 2. No effect of a high dosage of methionine on the urinary excretion of B_6 and its metabolites was observed in the study. 3. The role of vit. B_6 in the methylation of nicotinamide is shown by the increased urinary excretion of N^1-methylnicotinamide by human subjects, when given methionine plus pyridoxine but not when given only methionine.

1. Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, 1945, v157, 551.

2. Cerecedo, L. R., and Foy, J. R., *Arch. Biochem.*, 1945, v5, 207.

3. Kade, C. F., and Sheperd, J., *Fed. Proc.*, 1948, v7, 291.

4. Van Pilsum, J. F., and Berg, C. P., *J. Biol. Chem.*, 1950, v183, 279.

5. Roth, J. S., Allison, J. B., and Milch, L. J., *ibid.*, 1950, v186, 113.

6. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *ibid.*, 1946, v165, 55.

7. Cerecedo, L. R., Foy, J. R., and DeRenzo, E. C., *Arch. Biochem.*, 1948, v17, 397.

8. Linkswiler, H. M., Baumann, C. A., and Snell, E. E., *J. Nutrition*, 1951, v43, 565.

9. DeBey, H. J., Snell, E. E., and Baumann, C. A., *ibid.*, 1952, v46, 203.

10. Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, v146, 357.

11. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Nutrition*, 1947, v33, 561.

12. Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, v150, 401.

13. Ellinger, P., *Biochem. J.*, 1948, v42, 175.

14. Atkin, L., Schultz, A. C., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem.*, (Anal. Ed.), 1943, v15, 141.

15. Sarett, H. P., *J. Biol. Chem.*, 1951, v189, 769.

16. Huff, J. W., and Perlzweig, W. A., *ibid.*, 1947, v167, 151.

17. Linkswiler, H. M., and Reynolds, M. S., *J. Nutrition*, 1950, v41, 523.

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Effect of Meningococcal Endotoxin on the Immune Response.* (22032)

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Administration of sublethal quantities of various preparations of Gram negative bacterial endotoxin produce fever, leucocytosis preceded by leucopenia, depletion of liver glycogen, alterations in blood sugar levels, vasomotor disturbances and hemorrhagic necrosis of rapidly growing tumor tissues(1,2). In addition to these systemic reactions endotoxins produce profound effects on the host's natural defense mechanisms(3-5). An intravenous injection of suitable endotoxin establishes a state of "preparation" for the generalized Shwartzman phenomenon(6), so that a subsequent intravenous injection of endotoxin results in widespread hemorrhagic necrosis in the visceral organs of rabbits. The rabbit in this "prepared state" exhibits impaired activity of the reticuloendothelial system, as measured by rate of clearance of colloidal gold or chromic phosphate from the systemic circulation(3,5). Moreover, rabbits receiving a single intravenous injection of a bacterial free meningococcal culture filtrate prior to production of a pneumococcal skin infection show a marked decrease in resistance to the infection (4). The demonstration that phagocytic activity and resistance to experimental infections can be significantly altered by Gram negative bacterial endotoxins suggested that these compounds might alter the immune response. Our study was undertaken to determine the effect of various doses of meningococcal endotoxin on antibody response of rabbits to a single, purified foreign serum protein, crystallized bovine serum albumin.

Materials and methods. Hybrid albino rabbits of both sexes, obtained from a single breeding stock and weighing 2 kilos were used. Rabbits were maintained on diet of Purina rabbit pellets and were free of disease. The endotoxin employed was derived from a strain of meningococcus (44-B) supplied by Dr. Gregory Shwartzman, Mount Sinai Hospital, New York. The method for preparing the toxin has been described in an earlier communication(7). The antigen used was crystallized (BSA) bovine plasma albumin, lot #67009, obtained from Armour and Co. All toxin dilutions and antigen solutions were made up in pyrogen free saline just prior to use. Solutions for injection were prepared so that the final desired dilution of toxin plus the antigen, 15 mg BSA, were contained in total volume of 2 cc. Control animals received 15 mg of BSA in 2 cc volume of pyrogen free saline. Injections were given by way of marginal ear vein. Eleven days after antigenic stimulation, at a time when circulating antibody was presumed to be at its peak level

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TABLE I. Effect of Massive Dose of Meningococcal Endotoxin on Immune Response.

Group	Procedure†	Animal No.	Antibody content of serum, γn/ml	t	P
I	Bovine serum albumin, 15 mg Toxin 1-10 dilution	1652	30		
		1654	80		
		1656	60		
		1651	60		
		1658	36		
		1659	28		
		1661	10		
		1663	60		
			Mean 45 ± 7.62*		
II	Controls, bovine serum albumin, 15 mg	1676	28		
		1677	10		
		1678	28		
		1679	48		
		1680	60		
		1681	32		
		1682	10		
		1683	20		
		1684	50		
			Mean 31 ± 5.55		
I and II				1.259	>.10

* Stand. error of mean.
† Each group received 2 ml I.V. of injection solution made in pyrogen free saline.

(8), blood was withdrawn by cardiac puncture and serum separated, quick frozen at –70°C and kept in storage at –30°C until antibody content was determined. The quantitative precipitin technic for determining serum antibody content was a modification of the method described by Heidelberger and Kendall(9). The frozen serum samples were thawed 10 minutes at 37°C, then centrifuged at 3000 rpm at 4°C for one hour. The antibody content was determined at two separate times, a preliminary precipitation using ½ ml portions of serum and final determination using 1 ml of serum. Fresh standard antigen solutions were prepared for each precipitation. Preliminary tests were made to determine antigen concentrations that would precipitate more than ⅓ of the antibody and approach the equivalence point. The serum sample from each animal was precipitated with 4 different concentrations of antigen, incubated at 37°C for 1 hour and placed at 4°C for 7 days. The precipitates were broken up daily by gently swirling contents of each precipitin tube. At end of incubation period the tubes were centrifuged at 2500 rpm for one hour at 4°C, the supernate decanted and precipitates washed with 0.145 molar saline in the cold. This operation was repeated twice. Total nitrogen of precipitate was determined colorimetrically using Heidelberger-MacPherson modification of the Folin-Ciocalteu color reaction(10). Optical density was read at 650 μ on a Coleman Universal Spectrophotometer Model 14. Optical density was converted to nitrogen content by reading a standard calibration curve prepared with a highly purified preparation of human gamma globulin generously supplied by Dr. John T. Edsall, Harvard University. Supernatant analysis for antigen excess was performed in capillary tubes using a hyperimmune anti BSA serum. Capillary tubes were incubated at 37°C for 1 hour, placed at 4°C for 36 hours, then read for presence or absence of a precipitate. Final determinations were performed in identical manner using instead 1 ml serum samples. Antigen solutions for precipitations were made in 0.145 molar nitrogen free sodium chloride solutions. Nitrogen content of standard antigen solution was determined by the micro Kjeldahl method using an all-glass modification of the Markham distillation apparatus. Dilutions of a culture free meningococcal filtrate ranging from 1-10 to 1-10000 were selected to determine their effect on response of rabbits to a standard antigenic stimulation with crystallized bovine serum albumin.

TABLE II. Enhancement of Antibody Production by Meningococcal Endotoxin.

Group	Procedure†	Animal No.	Antibody content of serum, γn/ml	t	P
I	Bovine serum albumin, 15 mg Toxin 1-500 dilution	1013	96		
		1014	163		
		1015	44		
		1016	10		
		1017	50		
		1018	131		
		1019	240		
		1020	35		
			Mean 96 ± 25.74*		
II	Bovine serum albumin, 15 mg Toxin 1-100 dilution	1037	39		
		1038	99		
		1039	139		
		1040	67		
		1042	63		
		1043	49		
		1044	48		
			Mean 70 ± 12.13		
III	Controls, bovine serum albumin, 15 mg	1676	28		
		1677	10		
		1678	28		
		1679	48		
		1680	60		
		1681	32		
		1682	10		
		1683	20		
		1684	50		
			Mean 31 ± 5.55		
I and III				2.378	<.05
II and III				2.95	<.05

* Stand. error of mean.
† Each group received 2 ml I.V. of injection solution made in pyrogen free saline.

Results. Effects of Large Dose of Endotoxin on Immune Response. Intravenous injection of 2 cc of the toxin dilution (1-10) produced death within 24 hours after administration in 50% of treated animals. From these effects it was assumed that this dosage of endotoxin is the maximum feasible amount which can be employed to study the effect of meningococcal endotoxin on immune response. As shown in Table I, antibody levels of toxin-treated rabbits, although more variable and slightly higher than those of control rabbits, do not differ significantly from those of the latter group. No evidence of interference with antibody production by this amount of endotoxin was observed. In contrast, as shown in Table II, a smaller amount of endotoxin produced significant enhancement of antibody production.

Optimal Dosage of Meningococcal Endotoxin for Enhancement of Antibody Production. The effect of varying quantities of endotoxin on immune response was investigated. Dosages of endotoxin ranging from large amounts capable of producing death or severe prostration in a high percentage of rabbits (2 cc of a 1-10 dilution) through the range capable of inducing profound circulatory changes, preparation for generalized Shwartzman reaction, and provocation of local Shwartzman reaction, (2 cc of 1-100 and 1-500 dilution) to small dosages capable of inducing a pyrogenic effect but little other evidence of intoxication (2 cc of 1-1000 to 1-10000 dilution) were employed. Results are summarized in Fig. 1. Here it may be seen that while neither the large doses nor the smallest doses of endotoxin used produced significant enhancement of antibody production, increased antibody formation occurred when an intermediate dosage was employed. Optimum dilution of meningococcal endotoxin for this effect lies somewhere between 1-100 and 1-500. This is the amount of toxin

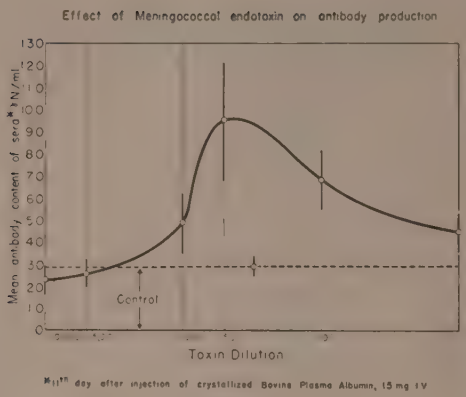

FIG. 1.

which produces profound circulatory changes, preparation for the generalized Shwartzman reaction and provocation of the local Shwartzman reaction in rabbits.

Quantities of toxin capable of producing a vigorous febrile response as well as characteristic alterations of granulocytes (1-5000 to 1-10000) do not act as an adjuvant to antibody production. Slight enhancement of antibody production was noted when 2 cc of the 1-1000 dilution were administered.

Discussion. Increased serum levels of antibody to bovine albumin are produced when a single injection of meningococcal endotoxin is given with it. Maximal enhancement of antibody production was demonstrated with quantities of toxin which are effective in preparing for generalized Shwartzman phenomenon. Massive doses of the toxin did not inhibit immune response as might have been expected from the demonstrated interference of endotoxin with reticuloendothelial function. Instead a slight statistically insignificant enhancement of antibody production was observed. Small quantities of endotoxin which consistently produce a pyrogenic effect and certain other reactions characteristic of this group of substances had no demonstrable effect on immune response of rabbits to the antigen.

Enhancement of immune response with quantities of meningococcal endotoxin employed was an unexpected finding. Cornwell and Good(3) studying the effect of substances such as cortisone, thorotrast and Gram negative bacterial endotoxins on phagocytic activity of reticuloendothelial system found that meningococcal endotoxin, in quantities which this study has shown to enhance antibody production, was most effective in decreasing the rate of clearance of radio colloidal gold from systemic circulation of rats and rabbits. Furthermore, it has been demonstrated that this same quantity of endotoxin results in a marked enhancement of susceptibility of rabbits to experimental infections with pyogenic microorganisms(4). On the basis of these observations it was predicted that a single injection of this dose of endotoxin would result in diminished production of antibody against an unrelated antigen administered simultaneously. Instead of diminishing antibody production the adjuvant effect herein described was observed. The basis for enhancement of antibody production by meningococcal endotoxin is not clear. It is possible that this action is related to the effect of endotoxins on phagocytic capacity of reticuloendothelial system previously mentioned. In support of this concept is the early demonstration by Lewis and Loomis(11) that certain dosages of trypan blue increased production of hemolysin against sheep erythrocytes. Other investigations, however, employing different blockading agents and even different dosages of trypan blue in association with varying immunization procedures, have demonstrated decreased rather than increased antibody production to result from "blockade" of the R. E. system(12,13).

Alterations in distribution of antigen based on changes in circulatory pattern in key organs or even alterations in vascular permeability might be responsible for the enhancement of antibody production herein reported. It seems possible that endotoxins might in some way activate the multipotent reticulum, the differentiation of which appears to be responsible for antibody formation in the rabbit (14). Unfortunately, discussion of the mechanism involved in reactions produced by Gram-negative bacterial endotoxins is hampered by lack of direct information concerning initial site of their action. Indeed, the biologic basis for the toxic properties of these sub-

stances has thus far not been elucidated. In general, however, it has been found that reactions produced by endotoxins derived from one group of Gram-negative bacteria are qualitatively indistinguishable from those produced by preparations from other unrelated Gram-negative bacteria(1,2,15). The phenomenon of enhanced antibody production appears to be no exception. In a recent communication, Johnson *et al.*(16) reported a marked enhancement of antibody production against egg albumin when this antigen was given in conjunction with a highly purified lipopolysaccharide antigen (endotoxin) derived from *Salmonella typhosa.*

The recent observations of Stetson(17) that reactions to Gram-negative endotoxins are strikingly similar to reactions produced with tuberculin in specifically sensitized rabbits are provocative and may represent a step toward better understanding of reactions to endotoxins from Gram-negative bacilli. These studies combined with the early demonstration by Lewis and Loomis(18) that guinea pigs infected with tubercle bacilli produce more antibody than do control uninfected animals suggest that perhaps a similar process is responsible for enhancement of antibody production in the two circumstances. Whether tuberculin in appropriately sensitized animals would act as does endotoxin in enhancing production of circulating antibodies is being investigated in our laboratory.

Many agents have been employed to augment production of circulating antibodies. Some of the more common include alum precipitation of antigen, tapioca, staphylococcus toxin and oil-in-water emulsions to which ground killed tubercle bacilli may be added (19,20). The mode of action of these various adjuvants has been generally attributed to production of prolonged inflammatory reactions associated with antigen retention at the site of injection(21-23). Halbert *et al.* (21) reported that with oleacious adjuvants appreciable quantities of antigen remain at the injection site for periods exceeding 22 weeks. However, it seems unlikely that the adjuvant property reported in our studies can be explained on the basis of prolonged retention of antigen at a local depot with consequent slow release and better antigenic stimulation. Rather, it seems likely to us that the ultimate explanation for the adjuvant effect of endotoxin lies in understanding the effect of endotoxin on the cellular system responsible for antibody production. This hypothesis has the advantage of permitting further investigation of the adjuvant action of endotoxin by morphological technics.

Summary. 1. The enhancement of antibody production against crystalline bovine serum albumin by the simultaneous injection of meningococcal endotoxin is reported. 2. An optimum dosage of endotoxin for production of the adjuvant effect is approximately the same as that required to prepare rabbits for the generalized Shwartzman reaction or to provoke the local Shwartzman phenomenon. 3. Failure of inhibition of antibody production by dosages of endotoxin capable of producing delayed uptake of radio active colloidal gold by the reticuloendothelial system is mentioned. 4. Possible mechanisms responsible for the adjuvant phenomenon are discussed.

1. Thomas, L., *Am. Rev. Physiol.,* 1954, v16, 467.
2. Bennett, I. L., Jr., and Beeson, P. B., *Medicine,* 1950, v29, 365.
3. Cornwell, S., and Good, R. A., *Anat. Rec.,* 1953, v115, 295.
4. Condie, R. M., Zak, S. J., and Good, R. A., *Fed. Proc.,* 1955, v14,
5. Gabrieli, E. R., personal communication.
6. Shwartzman, G., *Phenomenon of Local Tissue Reactivity,* New York, Paul B. Hoeber, Inc., 1937.
7. Thomas, L., and Good, R. A., *J. Exp. Med.,* 1952, v96, 605.
8. Dixon, F. J., Mauer, P. H., and Deichmiller, M. P., *J. Immunol.,* 1954, v72, 179.
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.,* 1935, v62, 467.
10. Heidelberger, M., and MacPherson, C. E. C., *Science,* 1943, v98, 63.
11. Lewis, P. A., and Loomis, D., *J. Exp. Med.,* 1926, v43, 263.
12. Perla, D., and Marmorston, J., *Natural Resistance and Clinical Medicine,* Little Brown and Co., Boston, 1941.
13. Stern, K., Spencer, K., and Farquhar, M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 126.
14. Kolouch, F., Good, R. A., and Campbell, B., *J. Lab. and Clin. Med.,* 1947, v32, 749.

15. Burrows, W., *Ann. Rev. Microbiol.*, 1951, v5, 181.

16. Johnson, A. G., Haines, S., and Landy, M., *Fed. Proc.*, 1954, v13, 499.

17. Stetson, C. A., Jr., *J. Exp. Med.*, 1955, v101, 421.

18. Lewis, P. A., and Loomis, D., *ibid.*, 1924, v40, 503.

19. Freund, J., *Ann. Rev. Microbiol.*, 1947, v1, 281.

20. Romon, G., *Rev. Immunol.*, 1937, v3, 193.

21. Halbert, S. P., Mudd, S., and Smoleuns, J., *J. Immunol.*, 1946, v53, 291.

22. Freund, J., Thompson, K. J., Hough, H. B., Sommer, H. E., and Pisani, T. M., *ibid.*, 1948, v60, 383.

23. Landsteiner, K., *The Specificity of Serological Reaction*, Cambridge, Harvard University Press, 2nd Edition, 1945.

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Binding of S^{35}-Labeled Sulfate in Serum of Rats as Studied by Filter-Paper Electrophoresis.* (22033)

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Filter paper electrophoresis of blood serum or plasma has been used to study the protein binding of radioactive substances(1-5). Dziewiatkowski(6) recently reported some observations on the association of sulfate sulfur with serum proteins. The present study was undertaken to extend our knowledge of sulfate binding by serum constituents. The results indicate that about 40% of the S^{35}-sulfate administered to Sprague-Dawley rats becomes bound to a substance or substances which migrate to the α_1-globulin region during electrophoresis on filter paper.

Materials and methods. Male Sprague-Dawley rats weighing 300-400 g were injected intravenously with 1200 μc of carrier-free $Na_2S^{35}O_4$. Blood was withdrawn from the heart 24 or 48 hours postinjection with a syringe containing 0.2 ml of mineral oil. The clot was spun down and the serum drawn off with a syringe. The S^{35} activity of the whole serum was determined by counting 100-μl samples in a gas flow proportional counter. Duplicate samples were spread on flat metal planchets and air dried for counting. The serum to be used for electrophoresis was stored at –30°C. Electrophoresis of 25 μl of blood serum placed on 3 by 29 cm strips of Whatman No. 1 chromatography filter paper was carried out in an apparatus similar to that described by Durrum(7). The samples were subjected to electrophoresis for 10 hours in a barbiturate buffer of ionic strength 0.05 and pH 8.6. Best results were obtained when the strips were immersed in the buffer and thoroughly blotted before the sample was applied. The current was maintained at 1.0 ma/strip, and the initial and final potentials were 300 and 150 volts, respectively. Following electrophoresis, the strips were dried at 60°C and developed in tetrabromophenolsulfonphthalein according to the procedure of Durrum(7). The dried and stained strips were scanned with a white light used in conjunction with a Photovolt photometer, and the optical density was recorded at 3-mm intervals. A standard serum electrophoresis pattern was obtained by plotting the optical densities against the distances from the origin. As an indication of the presence of carbohydrates, some of the strips were restained by the periodic acid-Schiff (PAS) routine according to the method of Köiw and Grönwall(8). The radioactivity associated with the serum proteins was determined by cutting the strips into 6 to 24 mm segments. Long or short segments were used where the activity was low or high, respectively. The segments were taped to planchets, and the S^{35} activity was determined in a gas flow proportional counter.

In view of the low S^{35} activity present on the segments of filter paper, it was necessary to

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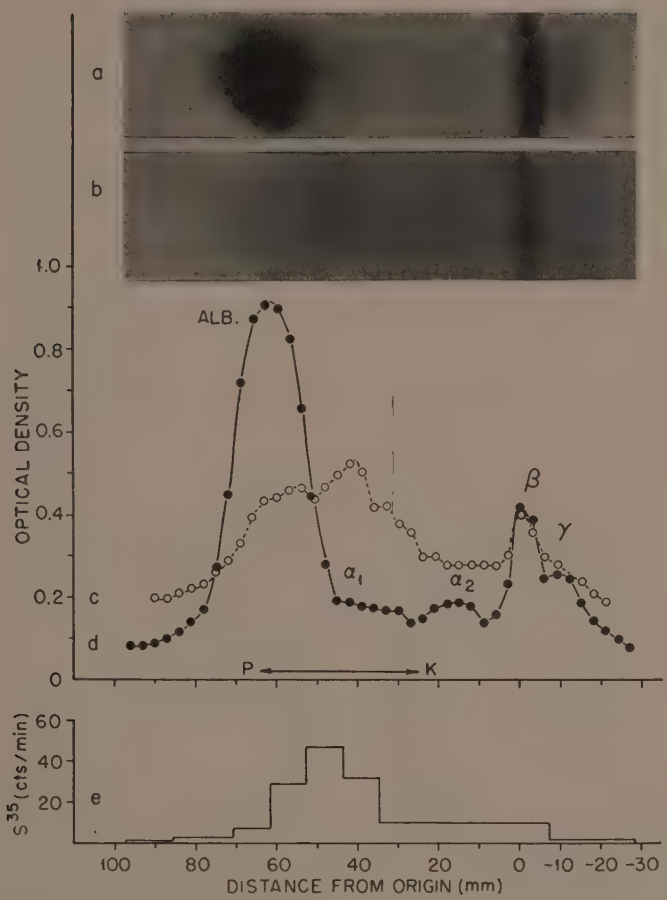

FIG. 1. Electrophoretic pattern of serum proteins as revealed by staining with tetrabromophenolsulfonphthalein (a) and periodic acid-Schiff routine (b). Graph (c) represents the optical densities of (b), and graph (d) depicts the optical densities of (a). Corresponding S^{35}-sulfate activities on the strip are shown in graph (e). S^{35} activity included between points P and K is referred to in the text as the activity peak.

calculate a counting time of sufficient length to insure the significance of the sample counts. A background count was established by averaging the background counts for 31 days. The daily counting periods ranged from 5-20 min., and a single day's value often represented an average of 2 or more such counts. The average value for each day always fell within one estimated standard deviation ($\sqrt{\bar{x}}$) of the mean background for the 31-day period. Since the background did not vary appreciably from day to day, it was considered to be a constant in calculating the sample counting time. It was decided to count each sample long enough so that the gross counts per minute minus 5 times the standard deviation of the gross counts per minute was greater than the background counts per minute. At the maximum sample counting time used, a value of 8 net cts/min. or greater was at least 5 standard deviations from the mean background count per minute. Thus any sample count greater than 8 cts/min. above background was considered to be significant.

TABLE I. Distribution of S^{35} Activity after Electrophoresis on Filter Paper of Serum from Normal Rats Injected Intravenously with $Na_2S^{35}O_4$.

Animal No.	Strip No.	Time post-inj., hr	Whole serum, cts/min./25 μl	Cts/min. in whole strip†	Cts/min. in peak†	% total counts recovered On strip	% total counts recovered In peak	% of recovered counts found in peak
640	91	24	3987	2134	1382	53.5	34.7	64.8
	106	24	3987	2370	1599	59.4	40.1	67.5
	161	24	3987	2270	1510	56.9	37.9	66.5
	159*	24	3987	2426	1596	60.8	40.0	65.8
641	92	24	4468	2384	1671	53.4	37.4	70.1
	100	24	4468	2925	2111	65.5	47.2	72.2
654	143	48	2581	1451	1006	56.2	39.0	69.3
655	144	48	2110	1345	914	63.7	43.3	68.0
645	118	48	1910	1359	844	71.2	44.2	62.1
					Mean:	60.0 ± 1.8	40.5 ± 1.3	67.6 ± 1.0

* This sample was dialyzed for 24 hr in cold against 3 changes of barbiturate buffer prior to electrophoresis.

† Corrected for self-absorption.

To determine what percentage of the whole serum activity remained on all or parts of the strip following electrophoresis, it was necessary to calculate a self-absorption factor. This factor was determined by comparing the activity of aqueous $Na_2S^{35}O_4$ dried on a planchet with the activity of the same solution dried on a disc of Whatman No. 1 filter paper attached to a planchet. The average of several determinations gave a self-absorption factor of 0.359. This factor was used to calculate the aforementioned percentages, but the activity values used to construct graph e in Fig. 1 are the original counts per minute uncorrected for self-absorption.

Results. A representative set of values obtained from the electrophoretic and S^{35} analysis of one serum sample is depicted in Fig. 1. The major part of the S^{35} activity coincided with a region on the strip which contained the α_1-globulin fraction. Occasionally it was observed that this maximum was shifted slightly into the ascending limb of the albumin region, but in no case did this maximum coincide with the albumin peak. The major part of the S^{35}-sulfate also coincided with a region on the strip which stained intensely with the PAS routine.

Calculations from the data for the S^{35} distribution on 9 strips are shown in Table I. It is seen that 60% of the whole serum activity was recovered on the strip, 40% of it being concentrated in a peak,† with the remainder distributed over the rest of the area occupied by the serum proteins (Fig. 1).

In one experiment (strip 159, Table I), the serum was dialyzed in the cold (2°C) against 3 portions of the barbiturate buffer during the 24 hours prior to electrophoresis. This procedure did not alter the pattern of distribution or the magnitude of sulfate activity on the strip.

To eliminate the possibility that the observed S^{35}-sulfate distribution on the paper strips resulted from independent migration of free sulfate ions, a serum sample to which $Na_2S^{35}O_4$ had been added *in vitro* was subjected to electrophoresis. The activity in this mixture was twice the amount found in the serum of rats injected with 1200 μc of $Na_2S^{35}O_4$, yet activity analysis of the stained strip showed no S^{35} present on any of the segments.

Discussion. In a paper on the effects of age on sulfate metabolism in the rat, Dziewiatkowski(6) reported that *in vivo* sulfate sulfur "becomes associated with serum proteins, mainly with albumin, and to a lesser extent with α- and β-globulins." The results of the present investigation showed that a major

† The S^{35} peak includes the activity present between the maximum point of the albumin curve and the low point anodal to the α_2-globulin region, *i.e.*, between points P and K in Fig. 1.

fraction of the serum sulfate is combined with a substance having the electrophoretic mobility of an α_1-globulin.

Sulfur is known to be present in 3 forms in the serum: one fraction is present as the sulfur in amino acids; another fraction is in the form of inorganic sulfate ion; and a third fraction is represented by several sulfuric acid esters, *e.g.*, heparin and indoxyl sulfuric acid. It has been shown by Tarver and Schmidt(9), and Boström and Jorpes(10) that administered S^{35}-sulfate does not become incorporated into the sulfur-containing amino acids of the rat, or becomes incorporated in negligible amounts (11). Our results, therefore, cannot be explained in terms of -S-S- or -SH groups.

The absence of activity on the paper strip following electrophoresis of serum mixed with S^{35}-sulfate *in vitro* confirms the recent report of Dziewiatkowski(6) that binding does not occur under *in vitro* conditions. In addition it indicates that the S^{35} in the α_1-globulin region did not result from the presence of free sulfate ions. Furthermore, the removal of free sulfate ions by dialysis of S^{35}-sulfate-labeled serum prior to electrophoresis resulted in no change in the amount of activity remaining on the strip after electrophoresis. These findings do not eliminate the possibility of a salt linkage between the sulfate ion and a protein, but the following preliminary experiments in this laboratory do not support such an hypothesis. After dialysis of S^{35}-sulfate-labeled plasma against a buffer of pH 12.0, 38% of the whole activity remained in the plasma, essentially the same amount (40%) as was found in the α_1-globulin region following electrophoresis of undialysed plasma at pH 8.6. At pH 12.0, few, if any, positively charged groups of a protein would be available for anion binding through a salt linkage. Even at pH 8.6, it is expected that the number of available groups of a protein capable of binding $SO_4^=$ would be small. In addition, anion binding would have to be quite strong to overcome the attraction of the sulfate ion to the anode during electrophoresis at pH 8.6. Although anion binding to a protein through a salt linkage might explain the present results, it seems more likely that the S^{35}-sulfate activity peak reflects the presence of sulfuric acid esters.

Proteins combined with sulfomucopolysaccharides such as chondroitin sulfate occur in many tissues. Protein-carbohydrate complexes such as the mucoprotein described by Winzler *et al.*(12) and an α_1-glycoprotein crystallized by Schmid(13) have been found in plasma. These 2 substances, isolated from human plasma and apparently identical, are acid although the composition of the acid groups of the carbohydrate moiety is unresolved(14). Schmid reported that his α_1-glycoprotein did not contain a sulfuric acid ester. Winzler *et al.*(12), and Weimer *et al.*(15) found, however, that after mild hydrolysis of their mucoprotein preparations a precipitate was formed by the addition of barium chloride to the hydrolyzate, suggesting that labile sulfuric acid groups had been removed from the mucoprotein. Winzler's mucoprotein has the electrophoretic mobility of an α_1-globulin at pH 8.4 (16), and contains approximately 30% carbohydrate(15). In the present studies it was observed that the S^{35}-sulfate activity peak corresponded to an area on the strip which contained PAS-positive constituents. Since the S^{35}-sulfate activity peak and the PAS-positive region appear to coincide electrophoretically with the mucoprotein described by Mehl *et al.*(16), it is possible that the carbohydrate moiety of this mucoprotein is esterified with sulfuric acid. A similar interpretation was previously suggested by Mehl *et al.* (16) to account for the observation of Winzler and Burk(17) that sulfur in excess of that required by cystine and methionine was present in a mucoprotein preparation from rat blood. With the use of human blood, Weimer *et al.* (15) made the same observation.

Summary. The binding of S^{35}-labeled sulfate in rat serum has been investigated by filter paper electrophoresis. About 40% of the whole serum S^{35}-sulfate activity was found to be combined with a serum constituent having the electrophoretic mobility of an α_1-globulin. Although the chemical identity of the labeled substance has not yet been established, the results of these experiments suggest that it may be a protein with a sulfonated carbohydrate prosthetic group.

1. Simon, N., *Science*, 1954, v119, 95.

2. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R., *Nature,* 1952, v169, 19.

3. Larson, F., Deiss, W. P., and Albright, E. C., *Science,* 1952, v115, 626.

4. Wallenius, G., *Scand. J. Clin. and Lab. Invest.,* 1952, v4, 24.

5. Winzler, R. J., and Notrica, S. R., *Fed. Proc.,* 1952, v11, 312.

6. Dziewiatkowski, D. D., *J. Exp. Med.,* 1954, v99, 283.

7. Durrum, E. L., *J. Am. Chem. Soc.,* 1950, v72, 2943.

8. Köiw, E., and Grönwall, A., *Scand. J. Clin. and Lab. Invest.,* 1952, v4, 244.

9. Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.,* 1939, v130, 67.

10. Boström, H., and Jorpes, E., *Experientia,* 1954, v10, 392.

11. Dziewiatkowski, D. D., *J. Biol. Chem.,* 1954, v207, 181.

12. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. and Lab. Invest.,* 1948, v27, 609.

13. Schmid, K., *J. Am. Chem. Soc.,* 1953, v75, 60.

14. Hughes, W. L., in *The Proteins,* 1954, vII, part B, 733-741.

15. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.,* 1950, v185, 561.

16. Mehl, J. W., Humphrey, J., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 106.

17. Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.,* 1944, v4, 417.

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Effect of Some Centrally Acting Drugs on Compulsive Circling. (22034)

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The group of signs and symptoms observed, following a unilateral intracarotid injection of di-isopropyl fluorophosphate (DFP) into rabbits, has been called the adversive syndrome (1) and the latter has been attributed to the anticholinesterase action of DFP(1-5). As a result of DFP administration, an asymmetric distribution of cholinesterase (ChE) activity is produced in the frontal cortex and caudate nucleus. The enzymatic activity is profoundly depressed on the injected side of the brain whereas the opposite side is relatively unaffected. This is especially marked in the right caudate nucleus which retains only 4% of its normal ChE activity. This inhibition permits asymmetric accumulation of acetylcholine (ACh) in the brain(6) which in turn may be directly responsible for the observed effects. It has been established in cats and monkeys that the adversive syndrome is the result of a biochemical lesion of the central nervous system(7). Although it is possible that peripheral receptors are more important in rabbits than in cats(7), many factors also point to a central mechanism for the production of the adversive syndrome in the former animal. First, the direct application of DFP to the peripheral vestibular receptors elicits no behavioral change in rabbits(8). Secondly, central stimulants can modify the forced circling behavior(5). Thirdly, a characteristic asymmetric brain cholinesterase pattern is found in all animals exhibiting the adversive syndrome, and this pattern is absent when compulsive movements are not observed (3,9,10). Finally a temporary or permanent reversal in the direction of the forced movements usually follows a minor convulsive seizure(2). The factors mentioned above appear to implicate the central nervous system as the primary site for the production of compulsive circling in rabbits. Previous work(5) showed that the adversive syndrome can be corrected by intravenous injections of small amounts of atropine sulfate after which the animal exhibits its normal mode of progression. When large doses (i.v.) of atropine are given the forced circling is reestablished, however, the animal turns in the opposite direction, toward the injected side.

In the present study a further analysis of

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TABLE I. Effect of Atropine-Like Drugs on the Adversive Syndrome (Dose of Drug, mg/kg).

Drug	Reversal after atropine*			Reversal without atropine		
	No. of exp.	Mean	Range	No. of exp.	Mean	Range
Scopolamine	5	.04	.02 -.08	4	.09	.06 -.12
Artane	8	.05	.02 -.10	6	.07	.02 -.12
Panparnit	6	.2	.1 -.3	5	.2	.1 -.4
WIN 2299	5	.015	.005-.02	5	.013	.005-.025

* Adversive syndrome was corrected by intravenous administration of atropine before experimental drug was injected.

this reversal is based on the ability of a variety of centrally acting drugs to produce this phenomenon. It is worth noting that the preparation to be described may be used as a bioassay for the atropine-like properties of drugs which act on the central nervous system. This bioassay is valuable because it establishes the drug action in terms of a behavioral response, namely the reversal in direction of the compulsive circling, in contrast to other assays which measure the drug effect on a single isolated organ or tissue.

Method. The adversive syndrome was produced in albino rabbits (approximately 2 kg) as described previously(5). The following drugs were used: atropine sulfate, scopolamine (hyoscine hydrobromide), Artane (trihexyphenidyl). Panparnit (diethyl aminoethyl-1-phenylcyclopentane-1-carboxylate hydrochloride), WIN 2299 (2-diethyl aminoethyl cyclopentyl-(2-thienyl) glycolate hydrochloride), Benadryl hydrochloride (diphenydramine hydrochloride), Diparcol (diethylaminoethyl-N-dibenzoparathiazine hydrochloride), LSD (d-Lysergic acid diethylamide), Reserpine (an alkaloid from Rauwolfia serpentina Benth), and Lergigan [N (2-dimethyl amino-2 methylethyl) phenothiazine]. All drug injections were made into a marginal ear vein. The *average values* (mg/kg) for a single intravenous injection of each drug were: atropine 0.2, scopolamine 0.02, Artane 0.02, Panparnit 0.1, WIN 2299 0.005, Benadryl 0.2, Diparcol 1.0, LSD 0.01, Reserpine 0.5, and Lergigan 0.2. Following each intravenous injection of a test drug, the rabbit was allowed to move freely on a rubber mat. The injections were continued at 2 to 6 minute intervals until the direction of the forced circling was reversed from that seen immediately after the administration of DFP. This reversal of forced circling was used as the end point in establishing the effect of the drug on the CNS. In those cases where no reversal of the forced movements was noted the injections were continued until the animals appeared to be in respiratory difficulty, in convulsions, or asleep. The last effect was noted only after Reserpine. In some cases atropine was administered prior to the test drug. This was done to obtain information on the synergism of atropine and the test drugs.

Results. The data obtained with atropine-like drugs are shown in Table I. In these experiments the amount of drug necessary to produce a reversal of the adversive syndrome was established in 2 groups of animals: 1) following atropine correction, and 2) without prior atropine administration.

The direction of the forced circling was reversed by a mean dose of 0.04 mg/kg of scopolamine when atropine was first injected in amounts sufficient to correct forced circling. However, 0.09 mg/kg of this drug was needed without atropine. The difference in these means was significant to beyond the 1% level when tested by the Student "t" test. Therefore, the pharmacological effects of scopolamine and atropine appear to be additive in our preparation.

The other 3 atropine-like drugs included in Table I, Artane, Panparnit and WIN 2299, required approximately the same dose of drug to produce a reversal in the direction of the forced circling whether or not atropine was first administered. In contrast to the results obtained with scopolamine, a synergism was not noted when these 3 drugs were used with atropine.

The effect on the adversive syndrome of some other drugs known to act on the CNS are presented in Table II. In general, these

TABLE II. Effect on Adversive Syndrome of Some Drugs Known to Act on the CNS (Dose of Drug, mg/kg).

Drug	No reversal			Reversal		
	No. of exp.	Mean	Range	No. of exp.	Mean	Range
Lergigan	3	2.7	1.2 - 3.5	5	1.8	1.2-2.8
Benadryl	8	3.0	1.0 - 6.6	2	.6	
Reserpine	5	4.6	3.0 - 6.0			
Diparcol	3	9.2	4.5 -12.0	1	5.0	
LSD	5	.05	.015- .09	1	.02	

drugs do not have a strong atropine-like action and were therefore administered to the rabbits without prior atropine treatment. Of the five drugs tested, Lergigan was best able to reverse the direction of the forced circling. It should be noted that a reversal was observed in 5 of the 8 animals studied.

Benadryl, a potent anti-histaminic reversed the direction of forced circling in only 2 of 10 animals tested. It was therefore far inferior to Lergigan in its ability to change the direction of forced circling. *Reserpine* does not affect the forced circling. Each of the 5 rabbits given this drug appeared to fall asleep with a scoliosis away from the injected side. A gentle tapping on the spine aroused the animal and it then circled about several times before resuming its sleeping posture. *Diparcol* reversed the involuntary movements in only one of the 4 rabbits tested. However, the mean dose needed was much larger than that of the other drugs listed in Table II. *LSD* was similar to Diparcol in its lack of a marked effect on forced circling. The direction of the compulsive movement was reversed in only one of the animals studied. In general, it appeared to be quite toxic when administered following the intracarotid injection of DFP.

Additional observations were made on ability of the listed drugs in Table II to correct the adversive syndrome. The corrected animal exhibits a normal behavioral pattern and may move in any direction. In a given animal the correction always occurs with a smaller dose of drug than that needed for reversal. In the present experiments this phenomenon was observed only with Lergigan and Benadryl. In the case of Benadryl 4 of the 10 rabbits were corrected, whereas 7 of 8 other rabbits studied were corrected by Lergigan.

Discussion. It has been previously reported (5) that small amounts of atropine correct the forced circling produced in rabbits by the unilateral intracarotid injection of DFP. This effect was referred to the power of atropine to block the action of accumulated ACh. On the other hand, large doses of atropine restored forced circling, but in the opposite direction. Since several CNS stimulants can also evoke a reversal in forced circling, it was suggested that this latter effect of atropine was due to a stimulant action. The fact that stimulation of the cortex may be important in producing a reversal is given further support from the observation that a mild convulsive seizure may be followed by such a change in the direction of the forced movements.

Of the drugs used in our study, the reversal in forced circling was most easily obtained with the atropine-like ones. However, it is apparent that the syndrome can be produced by a variety of drugs. These may be roughly divided into two categories: a) the CNS stimulants and b) the atropine-like drugs.

The suprabulbar vestibular system is complicated and the mechanism by which it is affected in the present preparation is unknown. However, two possible hypotheses may be suggested to explain the production of the reversed adversive syndrome: (a) the more nearly normal enzyme content of the left cortex may allow the intravenously injected drug selectively to stimulate that side more than the enzymatically depressed right side, thereby resulting in a reversal in the direction of forced circling, or (b) the production of convulsive activity on both sides of the cortex is followed by a bilateral depression in cortical activity. Both of these mechanisms would "neutralize" the original asymmetric activity of the cortex which follows the unilateral intracarotid injection of DFP. In the first case

(a) the reversal is then attributed to a stimulation of the left cortex by the injected drug and in the second (b) to the biochemical lesion in the right caudate nucleus which appears to be so severe as to preclude restoration of normal function by the administration of atropine or other drugs. It has been shown (11) that ablation of the right caudate nucleus (with or without destruction of the cerebral cortex) results in circling toward the right. The brain areas involved in the production of the adversive syndrome as well as the mechanisms by which drugs affect this system are at present the subject of a series of investigations in our laboratory.

The usefulness of the present method as a bioassay is demonstrated by the results obtained with the experimental drug WIN 2299. It is shown to be similar to Artane, Panparnit and Lergigan in its effect on forced circling. These drugs play an important role in the treatment of Parkinsonism. Clinical trials with WIN 2299 would seem to be profitable from our present results.

Summary. The intravenous injection of scopolamine, Artane, Panparnit, Lergigan or WIN 2299 into rabbits exhibiting the adversive syndrome evoke a reversal in the direction of the compulsive circling. Of the drugs mentioned, only scopolamine acts synergistically with atropine in causing this reversal. The usefulness of the procedure outlined as a bioassay for atropine-like drugs is discussed and two possible mechanisms by which the drugs studied produce this reversal are mentioned.

1. Freedman, A. M., and Himwich, H. E., *Am. J. Physiol.*, 1949, v156, 125.

2. Essig, C. F., Hampson, J. L., and Himwich, H. E., *Confinia Neurologica,* 1953, v13, 65.

3. Aprison, M. H., Nathan, P., and Himwich, H. E, *Am. J. Physiol.*, 1954, v177, 175.

4. Harwood, C. T., *ibid.*, 1954, v177, 171.

5. Nathan, P., Aprison, M. H., and Himwich, H. E., *Confinia Neurologica,* 1955, v15, 1.

6. Aprison, M. H., Nathan, P., and Himwich, H. E., unpublished observations.

7. Essig, C. F., Hampson, J. L., McCauley, A., and Himwich, H. E., *J. Neurophysiol.*, 1950, v13, 269.

8. Schiff, M., Esmond, W. G., and Himwich, H. E., *Arch. Otolaryngol.*, 1950, v51, 672.

9. Nathan, P., and Aprison, M. H., *Biochim. Biophys. Acta.*, 1955, v18, 150.

10. Aprison, M. H., Nathan, P., and Himwich, H. E., *Am. J. Physiol.*, in press.

11. Mettler, F., *The Diseases of the Basal Ganglia,* ARNMD, vXXI, 202, Williams and Wilkins Co., Baltimore, 1942.

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Practicability of Shipping HeLa Cell Suspensions for Use in Color Test For Poliomyelitis Antibodies.* (22035)

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The continuous cultivation *in vitro* of the human cancer cell (HeLa) by Gey and coworkers(1), and the important finding that these cells showed cytopathic effects in the presence of poliomyelitis virus(2), has made possible the use of many tests for poliomyelitis investigations without maintaining a monkey colony. Small laboratories are now better able to make such investigations providing ready access to a good supply of HeLa cells is available. The authors have previously reported their observations on the quantitative titration of 2000 human sera against 3 prototype strains of poliomyelitis virus employing as indicator system color changes of medium in tubes containing suspended HeLa cells(3).

This report concerns the practicability of employing suspensions of HeLa cells shipped to a laboratory for use in carrying out the same method.† The observations were undertaken in order to: (a) learn whether a small

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† Suspensions were obtained from George Washington Carver Fn., Tuskegee Institute, Ala., and may be procured from Dr. R. W. Brown there.

laboratory without special equipment could utilize this technic, (b) circumvent shipment of older cultures with heavy cellular growth since these may fall off during shipment, (c) reduce the cost of shipping numerous bottles, and (d) determine the practicability of shipping suspensions of cells for the initial source of a colony. It was observed by Youngner (4,5) that trypsinized monkey kidney cell suspensions could be shipped at the temperature of melting ice and were usable after a day or more. More recently, Melnick and associates (6), have described experiments in which monkey cell suspensions were prepared in India and flown to the United States.

Method. HeLa cell suspensions were prepared at Tuskegee Institute from young and old cultures which were fed shortly before preparation and shipment of suspensions. Cells were cultured in 250- to 500-ml bottles and dispersed with trypsin according to the method described by Scherer, Syverton, and Gey(2). For shipment the dispersed cells, 2-2.5 million per ml, were suspended in a medium of 40% human serum and 60% Hanks' solution. Penicillin and streptomycin were added to respective concentrations of 75 units and 75 μg per ml. The volume shipped was 100 to 125 ml in a glass container whose volume was 125 ml. The container was shipped at room temperature. Those suspensions made up on the afternoon of day 0 were received in our laboratory by noon of the following day (day 1). The suspension was stored at refrigerator temperature (4°C) and used usually on day 2 or day 3. The cell count was checked in a hemacytometer upon receipt of suspension and was within a reasonable range of deviation from the number reported shipped (minus 20%). On removal from the refrigerator the supernatant fluid was removed by suction and the cells washed in 40 ml of medium 199 containing 5% monkey serum and additional glucose(3). The low speed centrifugation for 10 minutes at 500 rpm was followed by removal of the supernate and cell washing for a total of 3 times. Finally, the cells were suspended in medium 199 containing 5% monkey serum with the additional glucose, adjusted so that each ml contained 200000 HeLa cells. Resulting suspension was then used in the colorimetric test(3).

TABLE I. Reproducibility of Serum Antibody Titers. HeLa cell bottle cultures vs. HeLa cell suspensions.

	Type I	Type II	Type III
Titers same*	120	112	103
Titers discrepant			
± 1 tube	50	57	62
± 2 tubes	7	15	14
± 3 or more tubes	2	1	6
Total No.	179	185	185
% reproducible (± 1 tube)	95%	91%	89%

* Highest dilution 1:1024.

Results. The technic outlined above has been employed to determine the antibody titers of 185 sera which have been titrated 7 months previously with cells shipped as bottle cultures, and trypsinized just before use to make suspensions for the test(3). Both tests were performed using a single tube per dilution per virus type with the following serum dilutions: 1:4, 8, 16, 64, 256, 1024. Table I includes the number of agreements and discrepancies which appeared when titers were compared. These titers were obtained in scattered tests, 13 different combinations of tests. Replicate titration results differed by no more than ±1 tube whether employing 2-fold or 4-fold dilution series. Sera which were recorded as agreeing by both methods ranged in titer from less than 1:4 to greater than 1:1024. Sera with titers recorded as greater than 1:1024 were not titrated further. Of 179 sera titrated against Type I virus, 120 were in agreement and 50 differed by ±1 tube (95% reproducible). A similar situation obtains with Type II and Type III antibody titrations with a reproducibility of 91% and 89%, respectively.

Forty-six shipments of cell suspensions were utilized in the titration of 4000 sera against Types I, II, and III poliomyelitis viruses. Control monkey sera of known titer, virus pools of known tissue culture infectivity and human sera of known antibody content have, when titrated by this method, yielded results in close agreement with known titers. Tech-

nically satisfactory titrations have been accomplished using cells sent from a central source and treated according to the method described above.

Summary. It has been found feasible to ship large numbers of suspended HeLa cells packed in a small container. Neutralization titers of 185 sera tested with 2 different types of shipped HeLa cells have yielded reproducible results. Under the conditions described the neutralizing capacity of 4000 serums has been tested against 3 prototype strains of poliomyelitis virus in a color test.

‡ Virus pools were obtained from Connaught Laboratories. Control human and monkey antisera were obtained from Dr. Herbert A. Wenner, University of Kansas.

The authors express deep appreciation to Dr. R. W. Brown for his thoughtful cooperation in planning this study.

1. Gey, G. O., Coffman, W. D., and Kubicek, M. T., *Cancer Research,* 1952, v12, 264.

2. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.,* 1953, v97, 695.

3. Lipton, M. M., and Steigman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 114.

4. Youngner, J. S., personal communication, 1954.

5. ———, *Ann. New York Acad. Sc.,* 1955, v61, 774.

6. Melnick, J. L., Rappaport, C., Banker, D. D., and Bhatt, P. N., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 676.

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Plasma Iodine[131] Levels as Diagnostic Tests for Lethal X-Radiation Exposure in Rats. (22036)

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At present there is no single, simple, diagnostic laboratory test for lethal exposure of man or experimental animals to ionizing radiation. In reviews of clinical diagnosis and treatment of radiation injury, Dunham *et al.*(1) and Cronkite(2) have pointed out that determination of the sequence of occurrence of various signs and symptoms is the principal method for establishing the severity of radiation exposure in man. Of various clinical laboratory tests, the white blood cell count appears to be of most value for prognosis(1,2). Relatively few diagnostic tests have been reported for experimental animals. Rosenthal(3) found an opalescence in the serum of rabbits exposed to lethal doses of x-rays and reported a correlation with lethality. Entenman *et al.* (4) confirmed this finding in rabbits but were unable to demonstrate it in other species. These workers also determined plasma phospholipid phosphorus (PLP) levels in a number of species of experimental animals and concluded that though they might be of value for prognosis of mortality in the dog, the PLP level did not serve as a good indicator of radiation injury "since in all species lethal doses of irradiation (LD_{50} to LD_{100}) are required to produce appreciable changes." Hayes and Hewitt(5) separated the plasma lipids of irradiated rabbits into various "classes" by ultracentrifugation and found an excellent correlation between increases in certain classes and mortality. Their method, however, has the disadvantage of complexity and is probably not adaptable to routine use. The uptake of radioactive iron by red blood cells of irradiated animals is known to be well correlated with radiation dose(6-9). The apparent disadvantage to this method is, that while it is sensitive to relatively low dose ranges, the relationship does not hold well in the lethal ranges since maximum depression occurs at less than lethal levels. Gershon-Cohen and Hermel(10) have recently reinvestigated the very early changes in the white blood cell count of irradiated rats and have concluded that such information may be of value in prognosis.

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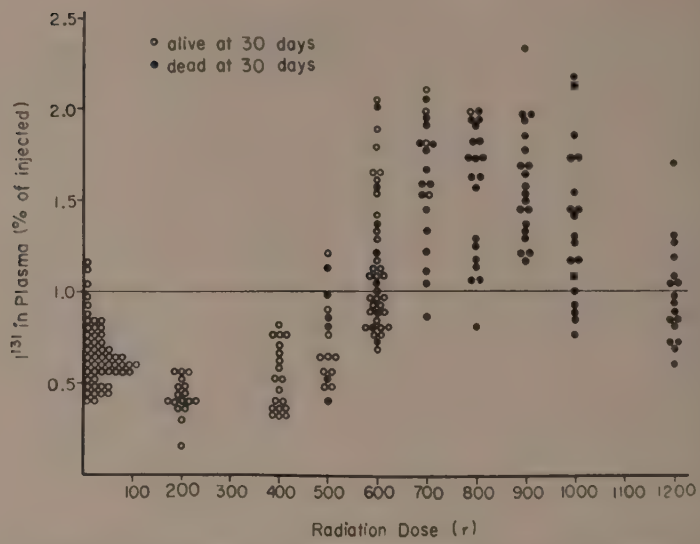

FIG. 1. Effect of various doses of x-radiation on iodine[131] levels in the plasma of rats.

In the first of a series of projected studies on the distribution of fission products in irradiated rats it was noted that there appeared to be a relationship between plasma iodine[131] level and the subsequent fate of the animal. This relationship was studied in more detail and is the subject of the present report.

Methods. A total of 242 adult male Sprague-Dawley rats was used. Since it was desirable to use as heterogeneous a group as possible, the rats selected weighed from 200 to 550 g and varied in age from approximately 3 to 18 months. They were housed 3 to a cage and given water and Purina Laboratory chow *ad libitum*. X-rays were delivered from a General Electric Maxitron unit using the following factors: 250 KVP, 30 ma, added filters of 1.0 mm Al and 0.5 mm Cu, TSD 50 cm, dose rate—115 r/min. The rats were exposed 3 at a time in a shallow lucite cage. The iodine[131], obtained from the Oak Ridge National Laboratory, was diluted with Krebs-Ringer phosphate buffer (pH 7) to a concentration of 5 μc/ml. Each rat received 1.0 of the solution intraperitoneally 48 hours following x-ray exposure. At that time suitable aliquots were set aside for counting at the same time that the plasma iodine determinations were made. Twenty-four hours after the injection of iodine (72 hours after irradiation), the animals were lightly anesthetized with ether and one ml of blood was removed into a heparin-wet syringe by cardiac puncture. The rats were allowed to recover, returned to their cages, and their survival was followed for 30 days. Autopsies were performed on animals dying the first few days following this procedure and any which showed evidence of hemorrhage from the puncture were excluded from the final tabulation of results. Blood obtained by cardiac puncture was placed in conical centrifuge tubes and the red cells were spun down. A 0.2-ml aliquot of plasma was then placed in a planchet containing 0.4 ml of 0.375 M NaOH and one drop of phenolphthalein solution. The solution was slowly evaporated to dryness under an infrared lamp and the iodine activity determined by beta counting under a thin-window Geiger-Muller tube connected to a conventional scaler. The iodine[131] activity in the total plasma volume was calculated as per cent of the injected dose by the formula:

$$\frac{(\text{Counts/min. in 0.2 ml plasma})\,(\text{body wt in g})\,(14.725)}{(\text{Counts/min. in 1 ml of inj. sol.})} = \%\ \text{of inj. dose in plasma.}$$

The factor 14.725 was obtained on the basis of the following considerations: (a) It was

TABLE I. Accuracy of Test in Prognosis at Various Doses of Radiation.

Radiation dose	No. rats	No. dead (30 days)	Positive values* Total	Positive values* Correct	Positive values* False	Positive values* Accuracy, %	Negative values* Total	Negative values* Correct	Negative values* False	Negative values* Accuracy, %	Combined accuracy, %
0	55	0	3	0	3	0	52	52	0	100	95
200	17	0	0	0	0	—	17	17	0	100	100
400	19	0	0	0	0	—	19	19	0	100	100
500	16	6	2	1	1	50	14	9	5	64	62
600	39	8	21	5	16	24	18	15	3	83	46
700	20	16	19	15	4	79	1	0	1	0	75
800	20	19	19	18	1	95	1	0	1	0	90
900	20	20	20	20	0	100	0	0	0	—	100
1000	19	19	14	14	0	100	5	0	5	0	74
1200	17	17	7	7	0	100	10	0	10	0	41

* Plasma I^{131} values in excess of 1% were considered "positive." Values of 1% or less were considered "negative."

assumed that there were 5.89 ml of blood/100 g body weight(8); (b) The hematocrit was assumed to be 0.50; (c) The counts/min. in the 0.2-ml sample of plasma were multiplied by 5 to convert to c/m/ml; (d) The entire equation was multiplied by 100 to convert to per cent,

$$\text{Thus: } \frac{5.89}{100} \times 0.50 \times 5 \times 100 = 14.725.$$

Results. Plasma levels of I^{131} (as per cent of injected dose) at various radiation doses are shown in Fig. 1. As radiation doses were increased in the range of 0 to about 900 r an increasing proportion of the rats showed relatively high levels of I^{131} in the plasma. At higher dose levels there appeared to be a reversal of the effect and the incidence of high values decreased.

Rats of the strain used in this study rarely die from radiation exposures of less than 500 r. At doses of 500 to 900 r, the incidence of lethality increases and at doses in excess of 900 r the incidence is nearly always 100%. The dose level of 500 r, then, can be taken as the point below which survival is reasonably certain and above which death may occur. From Fig. 1 it is apparent that I^{131} values of 1% or less were found in nearly all the control rats and rats exposed to less than 500 r. In the range of 500 to 900 r an increasingly greater percentage of the animals showed values in excess of 1%. For this reason, the 1% level was arbitrarily chosen to divide the results into "positive" (high) and "negative" (low) values. It was then assumed that positive values were prognostic of death and that negative values were prognostic of survival and the results were evaluated for accuracy as a possible diagnostic test. Table I lists the incidence of positive tests and lethality at various dose levels. In addition a tabulation of correct and incorrect predictions based on positive and negative I^{131} tests at each dose has been included. In this tabulation, death in a rat showing a positive value was considered a correct prediction as was survival in a rat showing a negative value. Death in rats showing negative tests or survival in rats showing positive tests were listed as false negatives or false positives, respectively. Percentage accuracy of positive and negative tests are shown separately and in the final column the over-all accuracy at each dose level is shown.

The test was not accurate at radiation doses in excess of 900 r, since a tendency to reversal of the effect occurred at these levels. This finding indicated that the test was valid over a restricted dose range and the doses in excess of 900 r exceeded this range. Accuracies at 1000 and 1200 r were computed in the same manner as for lower doses and, in the interest of completeness, are shown in Table I.

Discussion. Cronkite(2) has pointed out that for purposes of triage, a population exposed to ionizing radiation may be divided into the following groups: a. Sublethal exposure—recovery probable; b. Lethal exposure—recovery possible; c. Supralethal exposure—recovery improbable.

For purposes of evaluating the accuracy of the present test in dividing the sample rat population into these 3 groups, the population

was broken up as follows: a. Sublethal exposure—0 to 400 r; b. Lethal exposure—500 to 900 r; c. Supralethal exposure—over 900 r.

The assignment of the dosage ranges to these 3 groups was made arbitrarily based on the following considerations: Rats exposed to 0 to 400 r usually survive. In the range of 500 to 900 r some, but not all, of the rats usually die. With present methods of treatment of radiation injury, it is possible to increase survival in this range. Doses in excess of 900 r are uniformly fatal and survival is little influenced by present methods of therapy.

There was a total of 91 rats in the sublethal exposure group, all of which survived. Since 3 rats showed false positive values, the overall accuracy in this range was 97%. Tests were run on 115 rats exposed in the lethal (500 to 900 r) range. Twenty-two rats showed false positive and 10 showed false negative values for an accuracy of 72%. The principal source of error in this dose range was in false predictions of death (false positives). Since such errors would not be serious if the test had been used as an indication for instituting therapy, it may be permissible to ignore them and consider only the false negatives as errors. In this case, the accuracy in the lethal range was 91%.

Only 2 doses in excess of 900 r were employed, namely, 1000 to 1200 r. These doses may properly be considered supralethal. Of the 36 rats tested, 21 showed positive values and 15 showed negative values. All the rats died. The accuracy in predicting death, then, was only 58%. The increased incidence of negative values in this dose range may be of value in triage. The diagnosis of severe radiation illness was obvious from the severe weight loss, diarrhea, ruffled fur, etc. With present methods of treatment of radiation illness, it would undoubtedly have been impossible to prevent death in these animals. The combined clinical impression of severe damage and a negative I^{131} test may facilitate the diagnosis of supralethal exposure. Further investigation of these and higher dosage levels is, of course, required.

If the values for accuracy at doses in excess of 900 r are excluded, then the over-all accuracy of the test in the 0 to 900 r range was 83 to 95%, depending on whether false-positive values are acceptable.

It must be emphasized that the test as employed in the present study was entirely empirical. The mechanism by which the plasma levels of I^{131} were increased is not known. Refinements in technic or the use of different time intervals might increase the accuracy. At present, it may be stated that under the conditions of the experiment it was possible to diagnose exposure to sublethal and lethal doses of x-radiation with fair accuracy over a restricted dose range.

Summary. By determining plasma levels of trace amounts of radio-iodine in rats 24 hours after injection and 72 hours after x-irradiation with 0 to 900 r, it was possible to divide the animals into sublethal and lethal exposure groups with an accuracy of 83 to 95%, depending on whether a false diagnosis of lethal exposure is acceptable. There appeared to be a reversal of the effect on I^{131} level at doses in excess of 900 r, which might be of value in triage. At these dose levels, the diagnosis of severe radiation injury was obvious from the appearance of the animals and the combined clinical impression of severe damage and a negative I^{131} test might facilitate the diagnosis of supralethal exposure.

1. Dunham, C. L., Cronkite, A. P., LeRoy, G. V., and Warren, Shields, *J.A.M.A.*, 1951, v147, 50.

2. Cronkite, E. P., *U. S. Armed Forces Med. J.*, 1951, v2, 1019.

3. Rosenthal, R. L., *Science*, 1949, v110, 43.

4. Entenman, C., Neve, R. A., Supplee, H., and Olmstead, C. A., *U. S. Naval Radiol. Defense Lab. Rep. No.* Tr-2, 1954.

5. Hayes, T. L., and Hewett, J. E., *Univ. of California Rad. Lab. Rep.* No. 2790, 1954.

6. Hennessy, T. G., and Huff, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 436.

7. Furchner, J. E., and Storer, J. B., *Los Alamos Lab. Rep. No.* 1554, 1953.

8. ———, *ibid.*, No. 1849, 1954.

9. Belcher, E. H., Gilbert, I. G. F., and Lamerton, I F., *Brit. J. Radiol.*, 1945, v27, 387.

10. Gershon-Cohen, J., and Hermel, M. B., *Am. J. Roentgenol.*, 1954, v71, 846.

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Bacterial Allergy Increases Susceptibility to Influenza Virus in Mice.* (22037)

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We found previously that the vaccination of mice with *H. pertussis* vaccine increased their susceptibility to infection with several unrelated species of Gram-negative bacteria; such mice succumbed from a smaller number of live bacteria than normal mice. These results stimulated our interest in determining whether sensitization of mice with *H. pertussis* vaccine could make them more susceptible to virus. Experiments on this subject so far performed with influenza virus are described below.

Materials and methods. We used the CFW strain female mice of different ages: young mature females weighing about 22 g and weanlings weighing 10-12 g. Influenza virus A, strain WS, harvested in chick embryo allantoic fluid, with a hemagglutinin titer of 1:1280 was used for the intranasal inoculation of the mice. Sensitization was performed by the intraperitoneal injection of 15 billion cells of commercial *H. pertussis* vaccine. Experiments with sensitized mice were started 5-7 days after vaccination with *H. pertussis* and mice were observed for 2 weeks after the administration of the virus.

Results. Immunization with *H. pertussis* antigen considerably reduced the resistance to virus in mice of different ages. Normal and sensitized mice died within 5-10 days after an inoculation with the virus; however, the lethal dose of the virus for sensitized mice was much smaller than for normal mice of the same age group (Table I).

The young weanling mice which had been inoculated with the *H. pertussis* vaccine had become hypersensitive and could be shocked with the homologous antigen. The shocking dose of *H. pertussis* nucleoprotein had no effect on the normal, but killed 3 out of 6 of the sensitized weanlings. Adult mice injected with the same number of *H. pertussis* cells became sensitized to a higher degree; the same shocking dose of nucleoprotein produced up to 80-90% of anaphylactic deaths among the sensitized adult mice.

Four control groups of sensitized mice were used to demonstrate the mode of action of the influenza virus (Table II). When the influenza virus was inactivated and inoculated intranasally into sensitized mice it did not kill them, in contrast to the active virus, which in a dilution of 1:10000 given intranasally killed most of the sensitized mice. However, active virus, even undiluted, was well tolerated by sensitized adult mice if it was injected intraperitoneally.

Special studies were performed on the effect of sensitization by *H. pertussis* on the protection of mice by specific anti-viral antibodies. Immune serum was prepared by the injection of the same WS strain of influenza virus into rabbits. The immune serum had an anti-hemagglutinin titer of 1:5000. We studied the effect of the antibodies on the inactivation of the virus *in vitro* and *in vivo*. In

TABLE I. Susceptibility to Influenza Virus of Normal and Sensitized Mice of Different Ages.

Virus given .05 ml intranasally in dil.	Adult mice			Weanling mice		
	Normal	Sensitized	P value	Normal	Sensitized	P value
1: 1000	15/30	18/23	$.10 > P > .05$	31/35		
1: 10000	1/17	13/17	$P < .001$	16/22		
1:100000				6/19	15/18	$P < .01$

No. of dead mice in numerator; total No. of mice in denominator.

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The author wishes to thank Dr. J. F. McCrea for his help in the production and titration of virus and antiserum.

TABLE II. Different Pretreatments of Influenza Virus and Different Routes of Administration to Sensitized Adult Mice.

Material used for inoculum	Route of admin. and dilution	Results
Virus inactivated 30 min. at 56°C	Intranasally, .05 ml, dil. 1:100	0/ 6
Virus inactivated by incubation with 1% formalin at room temp., 24 hr	*Idem*	0/ 6
Allantoic fluid from non-infected eggs, same age as those used for harvest of virus	Intranasally, .05 ml, undil.	0/ 5
Active virus	Intraper., .2 ml, undil.	0/ 6
Idem	Intranasally, .05 ml, dil. 1:10000	13/17

the first series of experiments the virus in a dilution of 1:10 was mixed with an equal volume of antiserum diluted 1:10. After 30 minutes incubation at room temperature, .05 ml of the mixture was administered intranasally to each mouse. As a control, virus diluted in saline 1:20 was given intranasally in .05 ml volume. Normal and sensitized mice were used for this test and, since the results of both groups were the same, the data were combined. Virus inactivated with antiserum *in vitro* was not infectious for either normal or sensitized mice.

Different results were obtained in experiments in which mice received antiviral serum intraperitoneally and virus intranasally. In this case the protection of sensitized mice was very poor in comparison to normal mice. In one experiment some mice received immune serum undiluted; others in dilutions of 1:10 and 1:100; since protection with serum undiluted and diluted 1:10 was about the same in each group of normal and sensitized mice, the data were combined. Half of the mice in our tests were sensitized by *H. pertussis* vaccine as described above. In these experiments mice started to die 5-10 days after the injection of virus and were kept under observation for 2 weeks (Table III). An autopsy of the dead mice revealed lesions in the lungs typical of an influenza virus infection.

Discussion. The striking effect of the *H. pertussis* vaccine on the sensitization of mice and guinea pigs was described by the author and his collaborator in 1947(1). The development of increased sensitivity to histamine after vaccination with *H. pertussis* was observed by us in 1948(2), and since that time various studies of this phenomenon have been performed(3-5,8). In 1953 we observed(6) that vaccination with *H. pertussis,* which produced an immunity to the homologous organism, at the same time lowered the resistance of the mice to non-related infections with different Gram-negative bacteria and increased their susceptibility to *Shigella* endotoxin(7).

(1) Vaccination of mice of different ages with the *H. pertussis* antigen increased their sensitivity to the influenza virus (Tables I and III). The increase in the death rate in vaccinated mice in comparison to the normal animals of the same group could not be attributed to anaphylactic shock, but rather to the lowering of their resistance to an influenza virus infection. In vaccinated mice, as in normal animals, death occurred between 5-10 days after the administration of the virus. However, sensitized mice died from a smaller dose of virus. An autopsy of these mice showed that death was due to influenza infection with typical lesions of the lungs.

(2) Incubation with specific antiserum inactivated virus and rendered it non-infective for normal and for sensitized mice.

(3) Injecting antiserum intraperitoneally into normal mice produced considerable protection to intranasal inoculation of the virus.

TABLE III. Protection of Normal and Sensitized Mice by Antiviral Antibodies against Virus Administered Intranasally in Volume of .05 ml Diluted 1:20.

Serum inj. intraper. .2 ml	Mice vaccinated by *H. pertussis* vaccine	Normal (not vaccinated) mice	P value
Undil. and dil. 1:10	21/22	10/25	$P < .001$
Dil. 1:100	6/ 6	6/ 6	
No serum (control)	11/11	10/10	

On the contrary similar antiserum treatment gave very poor protection to the virus in sensitized mice. Apparently the state of hypersensitivity impaired the protecting qualities of the antibodies.

The observations presented in this report may have special interest for prophylactic work. Due to the present-day mass immunization of children with various bacterial antigens (including *H. pertussis*) and the possible increase of the number of cases of bacterial allergy, the regular test for the safety of a new vaccine on normal animals may not be adequate. Our studies offer a very simple technic for testing a new vaccine on hypersensitive mice.

Summary. Experiments with mice indicate that hypersensitivity induced by the *H. pertussis* antigen can increase susceptibility to influenza virus.

1. Parfentjev, I. A., Goodline, M. A., and Virion, M. E., *J. Bact.*, 1947, v53, 597, 603, 613.

2. Parfentjev, I. A., and Goodline, M. A., *J. Pharm. Exp. Ther.*, 1948, v92, 411.

3. Parfentjev, I. A., and Schleyer, W. L., *Arch. Biochem.*, 1949, v20, 341.

4. Parfentjev, I. A., *Yale J. Biol. Med.*, 1950, v23, 28.

5. ———, *Fed. Proc.*, 1955, v14, 474.

6. ———, VI Internat. Cong. for Microbiology, 1953, v6, ser. 17A, 99.

7. ———, *Yale J. Biol. Med.*, 1954, v27, 46.

8. ———, PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 297.

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New Olive Oil Emulsion for Lipase and New Observations Concerning "Serum Lipase". (22038)

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Emulsions prepared from olive oil, or similar fats, tend to separate on standing after short periods of time. For tests of lipase activity stable emulsions are required. In addition, there is in these tests a correlation between the degree of emulsification and the amount of lipase activity detected(1). In the present paper a simple method for preparing a stable emulsion of olive oil is described. With this emulsion the "lipase activity" of various sera was determined. In addition the following points were investigated: (a) the activation of serum lipase by Lima bean trypsin inhibitor, (b) a method for demonstrating and determining antilipase in serum, (c) other observations on lipase.

Methods. Preparation of a stable olive oil emulsion. The emulsion is a conventional olive oil-acacia mixture to which there is added 0.25% of a new hydrophilic colloid "Carbopol 934" (B. F. Goodrich Chemical Co.). Carbopol 934, having carboxyl groups, disperses in water to yield a low viscosity acid solution which on neutralization turns into a stable gel. Toluene (0.5%) is also incorporated into the emulsion to prevent bacterial growth. Control tests demonstrated that toluene does not inhibit lipase action. One gram Carbopol 934 and 200 ml of distilled water were mixed in Waring blendor until dispersion was complete (5 minutes). A solution of 5 g of acacia (McKesson, U.S.P.) in 100 ml of distilled water was added to the Carbopol solution; the mixture was stirred in a Waring blendor for 5 minutes and then filtered through glass wool. The blendor must be thoroughly washed at this time to remove the small particles of Carbopol which would interfere because Carbopol is a strong acid. The colloid mixture was then returned to the blendor, and 100 ml of olive oil (Fisher, U.S.P.) were added in a slow stream during 5 minutes with stirring. Without discontinuing mixing, N sodium hydroxide was then added dropwise until a pH of 7.00 was obtained. Usually 10.3 ml N sodium hydroxide

were required. Finally, 2 ml of toluene were added dropwise and homogenization was continued for 15 minutes. Air from the emulsion was removed in a desiccator with the aid of a pump. The emulsion may be kept in a refrigerator at 4° for several weeks without separating and without an appreciable increase in its titration blank. Brief shaking of the emulsion, however, is advisable before it is used. Digests prepared from this emulsion did not separate during the digestion period. When Carbopol was omitted from the emulsion the olive oil separated within a few hours. Cotton seed oil, as marketed in this country, cannot be used as a lipase substrate since it contains natural lipase inhibitors. *Phosphate Buffer.* 5.54 g of monobasic potassium phosphate and 3.41 g of anhydrous dibasic sodium phosphate were dissolved in distilled water and made up to 1000 ml. A few drops of toluene were added to prevent bacterial growth. The pH of buffer was 6.56. *Enzyme Preparations.* Human plasma cholinesterase was prepared from Cohn fraction IV-6 by Cutter Laboratories. It possessed 800-times the cholinesterase activity of whole plasma. Wheat germ lipase was obtained from Worthington Biochemical Corp.; the pancreatic lipase from Nutritional Biochemical Corp. Normal human sera were obtained from nonfasting blood donors (North Carolina Memorial Hospital); the other sera from nonfasting animals. *Crude Lima bean trypsin inhibitor (LBI).* This globulin fraction of Lima beans was prepared according to Tauber, Kershaw and Wright(2).

The lipase test. 1-ml portions of serum or enzyme solution were placed in 30 x 125 mm Pyrex test tubes. To each tube 5 ml of olive oil emulsion and 0.5 ml of phosphate buffer were added. The tubes were stoppered and shaken for 1 minute and then placed in a constant temperature water bath for 20 hours at 37°. Controls were prepared by adding the serum or enzyme solution to the olive oil emulsion-buffer mixture at the end of the digestion period. The control values were subtracted from the total values. At the end of 20 hours 3 ml of 95% ethyl alcohol and 2 drops of a 1% solution of phenolphthalein in ethyl alcohol were added. After mixing, the digests were placed in 30-ml beakers and titrated with 0.05 N sodium hydroxide. A 2-ml Koch micro burette was used to which soda lime tubes were attached to prevent absorption of CO_2 by the alkali. The mixtures were stirred with a glass rod after the addition of each drop of alkali. The solutions were titrated to a deep pink color, and the pH's (9.70-9.80) were checked with a Beckman Model G pH meter. All values are expressed in ml 0.05 N sodium hydroxide. *Kinetics of serum lipase.* Hydrolysis of the olive oil within 48 hours was fairly linear with respect to time. The following titration values (minus controls) were obtained: 24 hours, 1.55; 48 hours, 2.90; 72 hours, 3.92. The lipase activity was not proportional to increasing quantities of rabbit serum. 0.1 ml serum gave a value of 0.74; 0.2 ml gave a value of 1:00; and 1.0 ml gave a value of 1.65.

Keeping quality of serum lipase. Serum lipase appears to be a fairly stable enzyme. Different sera, when kept for 24 hours at 37°, lost none of their lipase activity when compared to specimens kept in deep freeze at –18°, and at 4° for the same length of time.

Serum antilipase. The presence of antilipase in inactive serum may be demonstrated by adding some of the inactive serum to some of the active serum. For instance, when 0.2 ml of inactive guinea pig serum was added to 1 ml of active rabbit serum the titration value dropped from 1.41 to 0.45 ml. In sera that have lipase activity the antilipase may be demonstrated by keeping an aliquot of the serum for 30 minutes at 56°. This destroys all but a trace of the lipase but not the antilipase. In the antilipase test, for convenience and because of its fairly high lipase content, dilute rabbit serum may be employed as the lipase source. The rabbit serum is diluted about 10 times in order to give a titration value of 1.2 ml with 0.05 N sodium hydroxide, after 20 hours. 0.4 ml of heated human serum is added to the dilute rabbit serum. The serum mixtures are made to 1.6 ml with 0.85% sodium chloride. After 30 minutes of interaction, the lipase test is applied. Inhibition of dilute rabbit serum by different quantities of "inactivated serum," however, is not quite proportional to the quantity of inacti-

vated serum employed. This is probably due to the presence of a trace of active lipase in the heated serum. *Inhibition of serum lipase by the antilipase of the same serum* may be demonstrated by adding 0.4 ml of heated rabbit serum (30 minutes at 56°) to 1 ml of 10-times diluted rabbit serum. Here the inactivated heated serum, containing the antilipase, brought about almost complete inhibition of the unheated serum lipase. The olive oil test was done in the usual manner. *Hemoglobin, a lipase inhibitor.* Hemoglobin is also a powerful inhibitor of serum lipase. Saline-washed rabbit red cells were frozen, thawed, laked with 20 volumes of distilled water and cleared by centrifuging. The lipase activity of 1 ml of rabbit serum was lowered from 1.54 to 0.73 when 0.2 ml of the laked rabbit red cell solution was added. For this reason sera obtained from hemolyzed blood cannot be employed in lipase assays.* *Lipase activity of different biological materials obtained with olive oil emulsion.* The following lipase values were obtained: human serum, 0.00-0.84 (average 0.22); rabbit serum, 0.50-1.70 (average 1.37); guinea pig serum, 0.00-0.25 (average 0.27); rat serum, none. Sixteen samples of human sera and 10 each of the other species, were employed. For other enzymes the following titration values were obtained: pancreatic lipase, 1.05; human plasma cholinesterase, 0.20; wheat germ lipase, 0.60. The digests contained 1 ml serum or 1 ml (20 mg) of enzyme solution, and 0.5 ml phosphate buffer. The digestions were carried out in a constant temperature water bath for 20 hours at 37°, except the pancreatic lipase-containing digest which was kept for 30 minutes in a constant temperature water bath. Only the water soluble portion (the filtrate) of the pancreatic lipase was used. The final pH of the serum digests was 7.00; the final pH of the other digests was 6.3.

Activation of serum lipase by crude Lima bean inhibitor (LBI). Table I shows typical experiments concerning the activation of serum lipase by the Lima bean inhibitor. This

TABLE I. Activation of Serum Lipase by LBI.

Type of serum	LBI, mg	Titration values, ml — Total	Minus control	Increase
Rabbit*	0	2.50	1.40	
"	1	3.00	1.90	.50
"	4	3.52	2.42	1.02
"	5	3.50	2.40	1.00
"	10	3.50	2.40	1.00
"	5†	2.77	1.67	.27
None	20	1.16	.06	
"	0	1.10 (control)		
Human*	0	1.58	.48	
"	5	2.35	1.25	.77
Horse (fasted)	0	1.20	.10	
"	5	1.95	.85	.75
Rat (fasted)‡	0	1.12	.02	
"	5	1.70	.60	.58

* Pool of 6 different specimens.
† Boiled 10 min.
‡ Pool of 18 specimens.
The digests contained 1 ml of serum. The LBI was dissolved in saline solution. The digests without LBI contained saline solution instead.

globulin fraction of Lima beans is a potent inhibitor of crystalline trypsin, a growth inhibitor of mammals(2), and an inhibitor of fibrinolysin(3). It was of interest to find out how lipase activity would be affected by LBI. It is seen in Table I that LBI is a powerful activator of human, rabbit, horse, and rat serum lipase. Boiling the LBI destroys some of its activity. LBI itself had no lipase activity. The activating action on serum lipase by the LBI cannot be explained at this time. Isolated serum proteins had no activating effect.

Summary. A stable olive oil emulsion has been described. Using this new emulsion the lipase activity of a series of biological materials have been studied. Sera of different mammals showed different lipase activities. Sera of some species had a wide range of activity, whereas, those of others had no activity. Wheat germ "lipase" splits olive oil only slightly. Highly purified plasma cholinesterase was almost inert indicating that this enzyme is not involved in fat digestion. Hemoglobin is a powerful inhibitor, and Lima bean inhibitor, a powerful activator of serum lipase. A method for demonstrating antilipase in serum has been presented. It is well known that normal human serum and the

* We are indebted to Dr. Paulo Seabra for calling this fact to our attention.

serum of most other mammals do not contain a specific lipase similar to pancreatic lipase. The hydrolysis of olein described in this paper is affected by another type of enzyme. For convenience the term lipase has been employed in this paper.

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1. Frazier, A. C., and Walsh, V. G., *J. Physiol*, 1933, v78, 467.

2. Tauber, H., Kershaw, B. B., and Wright, R. D., *J. Biol. Chem.*, 1949, v179, 1155.

3. Lewis, J. H., and Ferguson, J. H., *ibid.*, 1953, v204, 503.

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Variation in Response of Various Mouse Strains to Hexobarbital (Evipal). (22039)

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The use of inbred strains of mice, as an established experimental procedure in areas of medical research other than cancer, has been less extensive for various reasons, particularly because of the lack of information concerning characteristics of these established strains other than tumor type and incidences. Various investigators have determined some of the other characteristics these strains exhibit. Russell, Newfeld, and Higgins(1) have investigated the normal blood picture in 18 inbred mouse strains; Law, Morrow, and Greenspan (2) have investigated the liver glucuronidase activity in inbred strains; Tuba(3) has determined serum tributyrinase levels in 3 inbred strains; and Wragg and Spiers(4) have demonstrated inbred strain differences in response to adrenal cortical hormones.

The data herein reported is additional information on strain differences in mice, and is concerned with the response to a barbiturate drug, hexobarbital (Evipal), as measured by sleeping time.

Materials and methods. The various strains tested, shown in Table I, came from the production colonies of the National Institutes of Health. Initially, the investigation was concerned only with closely inbred strains, but because of the existence of some doubt about the uniformity of inbred strains in research of this sort(5), it was decided to extend the observations to additional strains in order to test other genetic situations. The first 10 strains listed in the table have been closely inbred by brother x sister matings, so they represent a genetic condition in which maximum homozygosity is to be expected. The CFW strain has been mated brother x sister in a large closed colony with no effort made to maintain close relationships between matings, so that many family lines are maintained parallel in each animal generation. Genetically, such a population will no doubt exhibit considerable homozygosity, but is expected to be more variable than the more closely inbred strains. The non-inbred Swiss strain has been random

TABLE I. Mean Sleeping Time in Minutes for Various Strains of Mice Injected with 125 mg per Kilo Body Weight of Evipal. All animals were males, 70-80 days old.*

Strains	No. of animals	Mean sleeping time	Stand. dev.
A/LN	25	48.44	4.17
DBA/2JN	64	46.83	4.22
A/HeN	25	41.25	1.92
BALB/cAnN	63	41.01	1.99
C57L/HeN	29	32.52	3.22
C57BL/6JN	37	32.26	5.71
C57BR/cdJN	25	31.93	5.51
C3HfB/HeN	30	22.02	2.99
BRSUNT/N	26	18.34	3.34
SWR/HeN	38	18.08	4.15
CFW	32	46.43	11.73
Swiss (non-inbred)	47	43.39	14.51

* The author wishes to thank Dr. Marvin Schneiderman, National Cancer Institute, for his advice on statistical procedures.

mated within a large closed colony, and hence represents considerable heterozygosity. All animals were 70-80 days old when tested, and only males were used. The barbiturate, Evipal, was injected intraperitoneally at a dose level of 125 mg per kilo of body weight. Sleeping time was measured in minutes from the time the injection was made until the righting reflex was restored. The test for significant differences between sleeping time means was made according to the method of Baten(6).

Results. Table I shows the results of sleeping time determinations in various strains of mice. As the data show, strain differences in response to the drug are evident. The A/L and DBA/2 strains sleep the longest with no significant difference existing between the 2 sleeping times. Likewise, the A/He and BALB/c strains fall into a second group with no difference between the 2, but both differing significantly from the A/L and DBA/2 strains. The 3 C57 strains fall into a third group with no difference between the 3, but differing significantly from the first 4 strains. The C3HfB is similar in sleeping time to the BRSUNT and SWR, but differs significantly from these 2 strains. The CFW and Swiss have long mean sleeping times, comparable with the A/L and DBA/2 strains.

The uniformity of the distributions of the various strains is of particular interest. An examination of the standard deviations will readily show that the distributions of the inbred strains are considerably less variable than those of the CFW strain, and it in turn is less variable than the non-inbred Swiss. This latter strain was quite variable, ranging from one animal that was completely unaffected to 2 animals that slept 97 minutes. Thus, 3 different levels of genetic homogeneity have exhibited 3 different levels of variability, with the closely inbred strains showing minimum variation at this particular injection level. Whether this will hold true for other injection levels is not known. However, in this regard, preliminary trials with injection levels of 25 mg, 50 mg, and 75 mg per kilo of body weight showed considerable variation in response by all the strains. More complete information on this point is not yet available.

Discussion. The significance of the differences in mean sleeping time between the various strains tested is not apparent at this time. Whether this indicates differences in sensitivity to hexobarbital, or differences in the rate of biotransformation is yet to be determined. Quinn, Axelrod, and Brodie(7) have found an inverse relationship between the rate of biotransformation of hexobarbital and the duration of its hypnotic effect in a number of species. Perhaps a similar relationship exists in these various strains of mice. The fact remains, however, that strains do differ in their response to this drug, and these differences are consistent and uniform for the strain. Therefore, a genetic mechanism is probably involved.

Strain differences in response to various drugs could be of considerable importance in routine pharmacological testing of drugs, or in basic studies of drug metabolism systems. It is quite likely that one approach to the study of metabolic pathways in small mammals can be made by the use of genetically uniform animals in combination with pharmacological technics.

The demonstration of strain differences in response to such a physiological condition is interesting from a genetic standpoint, for it supports the concept of the uniformity of response of closely inbred strains, a concept that has been the backbone of the creation and use of inbred strains for many years. Furthermore, the demonstration that the uniformity of response corresponds to the theoretical state of homozygosity, supports the concept even more strongly. However, in this regard, recent work by McLaren and Michie(6) using pentobarbital sodium (Nembutal), indicated the inbred strain tested was just as variable as an F1 hybrid of that strain and as a non-inbred strain. The physiological action of these 2 chemically similar drugs has always been considered similar, so whether this discrepancy in results is due to an actual difference in action, or difference in dose levels, or differences in the animals used cannot be determined at this time. Certainly additional work on drug metabolism in the various inbred and non-inbred strains of mice is indicated.

Cooper and Brodie(8) have recently demon-

strated an enzyme system localized in the microsomal fraction of liver cells, which oxidizes hexobarbital to keto-hexobarbital. The investigation of the activity of this enzyme system in these strains is now underway.

Summary. 1. Significant differences in sleeping time have been demonstrated between various strains of mice, inbred and non-inbred, when injected with 125 mg per kilo body weight of hexobarbital. 2. Three different levels of genetic homozygosity were represented in the various strains tested. The most closely inbred strains (A/L, DBA/2, A/He, BALB/c, C57L, C57BL/6, C57BR/cd, C3HfB, BRSUNT, SWR) showed the least variation, the less closely inbred strain (CFW) showed more variation, and the non-inbred Swiss strain showed the most variation.

1. Russell, E. S., Newfeld, E. F., and Higgins, C. T., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 761.

2. Law, L. W., Morrow, A. G., and Greenspan, E. M., *J. National Cancer Inst.*, 1952, v12, 909.

3. Tuba, J., *Cancer Res.*, 1952, v12, 113.

4. Wragg, L. E., and Spiers, R. S., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 680.

5. McLaren, A., and Michie, D., *Nature*, 1954, v173, 686.

6. Baten, W. D., 1938, Elementary Mathematical Statistics, New York, John Wiley & Sons, Inc.

7. Quinn, G. P., Axelrod, J., and Brodie, B. B., *Fed. Proc.*, 1954, v13, 395.

8. Cooper, J., and Brodie, B. B., *J. Pharm. Exper. Therap.*, 1954, v110, 12.

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Blood ACTH Levels Following Subcutaneous Injections.* (22040)

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The adrenocorticotropic hormone (ACTH) is known to disappear rapidly from the circulating blood. Van Dyke *et al.*(1) have estimated the average life of the ACTH molecule in the circulation to be approximately 17 minutes, and half-life values of about 5 minutes have been estimated for ACTH administered intravenously to intact rats(2,3). The half-life of endogenous ACTH in the adrenalectomized rat has been reported to be approximately one minute(4). A rapid rate of disappearance of ACTH from the circulation is also indicated by clinical studies. Intravenous infusion of ACTH over a prolonged period brings about a much greater adrenal response than the single injection of a comparable dose of the hormone(5). It becomes of interest, therefore, to establish the rate at which exogenous ACTH disappears from the circulation following a single extravascular administration.

In these studies a large dose of ACTH was administered subcutaneously to hypophysectomized rats and blood ACTH levels were estimated at various intervals after treatment.

Method. Male hypophysectomized rats were obtained from the Hormone Assay Laboratories, Chicago, and used within a 24-hour post-operative interval. An ACTH standard dose response curve was prepared for this study based on accumulated data representing

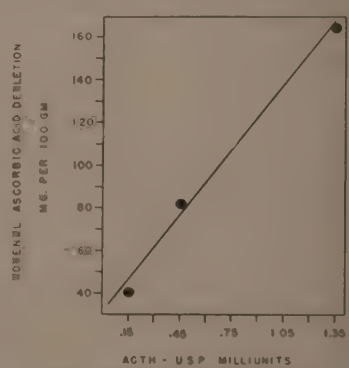

FIG. 1. ACTH standard dose-response curve. Each point represents the mean response of 25 hypophysectomized rats 1 hr after intrav. injection with U.S.P. ACTH Reference Standard.

* The authors acknowledge the helpful assistance of Mr. William Watson and Miss Joan Michalski.

TABLE I. Depletion of Adrenal Ascorbic Acid in Recipient Rats following Injection of 4 ml of Blood from Donor Rats Previously Injected with 10 U.S.P. Units of ACTH.

Min. after subcut. inj. of donor rats	No. of rats	Adrenal ascorbic acid depletion (mg/100 g) of recipient rats 1 hr after intrav. inj.	Blood ACTH of donor rats (mU/4 ml)*
1	6	77	.44
2	6	90	.56
4	4	134	1.00
8	5	119	.88
10	3	106	.76
16	6	123	.92
20	3	43	.12
32	3	69	.36
40	3	58	.32
80	3	10	.00

* Obtained by interpolation of column 3 using Fig. 1.

25 rats at each point injected intravenously with U.S.P. ACTH Reference Standard (Fig. 1). Measurements of blood ACTH were made by the technic of Sayers and Burke(6). Hypophysectomized rats, 250 g, were injected subcutaneously with 10 U.S.P. ACTH units in 0.5 ml water. At various time intervals following treatment 4 ml of blood from a previously injected rat were collected from the abdominal aorta into a heparinized syringe. The blood was immediately injected into the jugular vein of a recipient 100 g hypophysectomized rat. The donor rat was then discarded. Individual rats were used for each blood collection. Adrenal ascorbic acid of recipient animals was determined 1 hour after the transfusion. These values were subtracted from uninjected control values to estimate adrenal ascorbic acid depletion. Blood ACTH values were obtained by interpolating the depletion values on Fig. 1.

Results. Table I presents the ascorbic acid depletion values found in recipient rats receiving 4 ml of blood from donor animals.

The concentration of ACTH in each ml of rat blood (obtained from Table I) is shown graphically in Fig. 2. Detectable ACTH activity is found in the blood within one minute after subcutaneous injection.

The blood ACTH level rises rapidly and reaches a peak concentration between 4 and 8 minutes after injection. After 20 to 40 minutes the blood ACTH is almost back to zero and 80 minutes after injection no detectable activity is found.

In a separate experiment, adrenals were removed from the donor rats at the time that blood sampling took place. The adrenal ascorbic acid depletion was determined and is shown plotted with blood ACTH levels in Fig. 3. Significant depletion of adrenal ascorbic acid in the donor rats does not occur until about 28 minutes after the peak level of ACTH in the blood is reached.

Discussion. The present data indicate that exogenous ACTH, even when administered subcutaneously in a large dose rapidly disappears from the blood. Endogenous ACTH, too, disappears quickly from the blood after ether anesthesia(6) and after scald(7).

Pincus *et al.*(8) showed that up to 70% of added ACTH activity is lost within 1 hour from blood. Since such *in vitro* inactivation may be prevented by previously heating the blood(8), it is likely that at least some of the disappearance of ACTH from blood *in vivo* may be due to enzymatic inactivation.

The time relationship between ACTH administration and adrenal ascorbic acid depletion has been the subject of many studies (9,10), but this is believed the first investigation where the actual ACTH concentration in the blood was correlated with its corresponding adrenal ascorbic acid depletion. This is significant since it shows that despite an unphysiologically high ACTH blood concentration a 28-minute lag in the adrenal response still occurs.

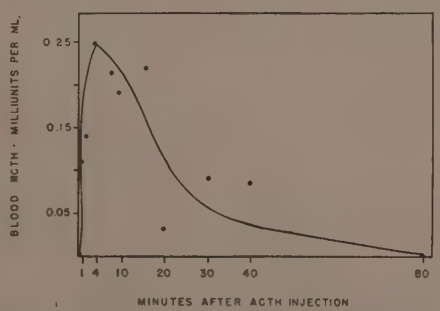

FIG. 2. Blood levels of ACTH in hypophysectomized rats after a single subcut. injection of 10 U.S.P. ACTH units. Each point represents mean response of 3 to 6 animals.

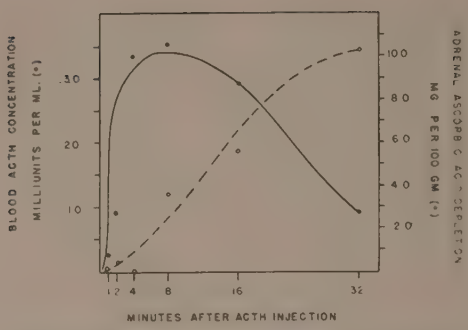

FIG. 3. Simultaneous measurement of blood ACTH concentration and adrenal ascorbic acid in hypophysectomized rats treated subcut. with 10 U.S.P. ACTH units. Each point represents mean response of 3 animals.

The highest blood concentration of ACTH observed in this study (Fig. 3) was 0.35 milliunit per ml blood. The blood volume of a rat is estimated to be 6 ml per 100 g(11,12). In the 250 g rats used in these experiments a total of only 6.1 milliunits, therefore, appears to be present in the circulation at the time of the highest blood concentration out of 10,000 milliunits injected. This indicates massive inactivation of the hormone at probably both the site of injection and after it enters the circulation.

Summary. The rate of disappearance of exogenous ACTH from the blood in the hypophysectomized rat has been determined. Following subcutaneous injection of 10 U.S.P. ACTH units detectable blood ACTH activity was found within 1 minute. A peak concentration of 0.25 to 0.35 milliunit per ml blood took place 4 to 8 minutes after injection. No detectable activity was found after 80 minutes. Significant adrenal ascorbic acid depletion did not occur until about 28 minutes after the peak concentration of ACTH in the blood was reached.

1. Van Dyke, D. C., Simpson, M. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, 1950, v163, 297.

2. Greenspan, F. S., Li, C. H., and Evans, H. M., *Endocrinol.*, 1950, v46, 261.

3. Gemzell, C. A., Van Dyke, D. C., Tobias, C. A., and Evans, H. M., *ibid.*, 1951, v49, 325.

4. Sydnor, K. L., and Sayers, G., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 729.

5. Renold, A. E., Forsham, P. H., Maisterrena, J., and Thorn, G. W., *New England J. Med.*, 1951, v244, 796.

6. Sayers, G., and Burke, R., *Fed. Proc.*, 1955, v14, 131.

7. Sayers, G., *J. Clin. Endocrinol.*, 1955, v15, 754.

8. Pincus, G., Hechter, O., and Hopkins, T., *ibid.*, 1952, v12, 920.

9. Sayers, G., Sayers, M. A., Fry, E. G., White, A., and Long, C. N. H., *Yale J. Biol. Med.*, 1944, v16, 361.

10. Sayers, G., and Sayers, M. A., *Ann. N. Y. Acad. Sci.*, 1949, v50, 522.

11. Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *J. Biol. Chem.*, 1933, v99, 457.

12. Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, v141, 695.

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Pathogenesis of Fatal Encephalomyocarditis (EMC) Virus Infections in Albino Rats. (22041)

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This paper presents a study of encephalomyocarditis virus infections, carried out by means of virus titrations and histopathologic studies of various organs of albino rats moribund from disease. EMC virus can produce paralysis and fatal encephalitis in albino rats although other species of rats and mongooses, when inoculated with this agent, appear to die principally from myocarditis(1). The subject has additional interest in that previous investigators(2-6) have reported only inapparent infections in albino rats.

Materials and methods. Experimental animals. Albino rats were random bred. Some were originally obtained from Makerere College Medical School and others from Veterinary Research Laboratory, Kabate, Kenya. Hooded rats, which originated at Lister Institute, Elstree, England, were employed for a few experiments. Mice were of the Swiss albino strain. *Virus.* Of 3 strains of EMC virus employed, 2 had been isolated from animals in monkey runs of the Virus Research Institute. The principal one we used was originally obtained from an infected baboon and had been carried through 6 intracerebral passages in mice; the second strain was isolated from a rhesus monkey and had been passaged 16 times in mice; the third strain was one originally ioslated from a chimpanzee in Dania, Florida(7), and had been passed 78 times in mice. This last strain was obtained from Dr. Karl Habel, National Institutes of Health, Bethesda, Md. The rhesus and Florida strains were studied for comparative purposes. *Virus isolations from inoculated animals.* Ten percent tissue suspensions in meat infusion broth containing penicillin and streptomycin were centrifuged at 4000 rpm for ½ hour prior to intracerebral inoculation of 0.03 ml into mice 5 weeks of age. For titrations, groups of 4 mice were used for each 10-fold dilution. Blood obtained by cardiac puncture was heparinized to prevent clotting. Urine, aspirated from the bladder at necropsy, was obtained from only a few animals. Samples of gut were removed routinely from the lower ileum and laid open and flushed free of intestinal contents in 3 changes of broth. Formed feces were obtained by incision of the rectum. Storage for all specimens was at –20°C. Under these conditions, little or no diminution of virus titer in frozen specimens appeared to take place within 4 months. A majority of suspensions, nonetheless, were titrated in mice shortly after being harvested. Inoculated mice were then observed for 8 days for paralysis and death. Specificity of illness induced was judged by a characteristic incubation period of 3-5 days and the clinical picture, of which paralysis of the hind legs was a cardinal feature. Fifty percent end-points were calculated by the method of Reed and Muench(8). *Neutralization tests.* Spot identifications of viruses isolated were made by means of neutralization tests performed with normal and EMC-immune rabbit sera. The antiserum was prepared by immunization of rabbits with brain suspensions of mice injected with the Florida strain of EMC virus. All sera were inactivated at 56°C for ½ hour prior to mixing with an equal volume of virus suspension that would give final dilutions containing 100 or more LD_{50} of virus. Serum-virus mixtures were inoculated into mice intraperitoneally in volumes of 0.1 ml after ½ hour of incubation at room temperature.

Results. EMC virus (baboon strain) was carried through 15 passages in albino rats 3 to 5½ weeks of age by intraperitoneal inoculation of 10% suspensions of brains of ill rats. Repeated passage did not result in any ob-

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TABLE I. Virus Titrations of Various Organs in Albino Rats Infected with EMC Virus. Rats were sick 24 hours or less when sacrificed.

Rat	Strain of virus	Age inoc. in days	Blood	Brain	Fem. mus.	Heart	Spleen	Ileum	Feces	Urine	Incubation period, days
113W	Baboon	5	4.6*	7.7	4.5	2.7	2.3	—	—	—	2
114W	"	11	3.7	7.5	5.3	3.5	3.3	4.5	0	0	3
47W	"	25	<1	4	1	1	1	0	0	—	3
117W	"	32	<1	5.2	0	1	1.5	0	0	—	4
133W	"	72	0†	4	0	<1	<1	0	—	—	9
127W	Florida	30	0	4.5	1.4	0	1.5	0	0	—	4
128W	Rhesus	29	0	5.5	0	2.5	0	0	0	—	5

* Titers expressed as inverse log of dilution killing 50% of mice inoculated.
† 0 = No virus isolated from 1:10 suspension.

served change in virulence. Although rats of all ages might become paralyzed and die, susceptibility, which was 100% in suckling animals, became increasingly unpredictable in rats over 3-4 weeks of age. Severe crippling of one and frequently of all 4 extremities gave rats a helpless appearance and occasional rats had a hemorrhagic, watery exudate along the eyelids. Death usually supervened in one to 3 days. The incubation period was 2-3 days in rats under 2 weeks of age, and 4-6 days, rarely up to 11 days, in older rats. Table I presents comparative titrations of various tissues from albino rats of increasing ages which were killed when moribund. All, except for 47W, were given the same inoculum of 12th passage rat brain intraperitoneally. Suckling rats (113W and 114W) had considerable amounts of virus in all organs tested with highest titers in brain tissue, whereas the older rats (47W, 117W, and 133W) had little recoverable virus from any organs except brain. Seventeen other rats, from 22 to 39 days of age and representing 4th to 14th passages of EMC virus, were also sacrificed when moribund and studied by methods similar to those just described. Although titrations in many cases were limited to blood, brain, and femoral muscle, titers obtained were essentially the same as those obtained for rats 47W, 117W, and 133W (Table I). None of these older rats exhibited the widespread distribution of virus encountered in suckling animals. Two rats, 3 to 4 weeks of age, inoculated intraperitoneally with 13th-passage brain suspensions, developed paralysis of the hind legs within 4 days. Brains, when harvested and pooled, had a titer of $10^{-4.5}$. Lumbar spinal cord from one rat had a titer of $10^{-2.5}$ and from the other of $10^{-2.8}$.

Principal histologic changes encountered in limited examinations of sick rats were observed in the brain. In brains of 3 rats examined, lesions consisted of scattered foci of degeneration and cellular infiltration with lymphocytes and macrophages. No study was made of the localization of these lesions. Lesions were also found in spinal cords from 3 rats examined. These included focal hemorrhages, perivascular infiltration with polymorphonuclear leucocytes, lymphocytes, macrophages, and degeneration and loss of nerve cells. Definite myositis was found in both of two rats examined. These rats, inoculated intraperitoneally at 3-4 weeks of age, had developed crippling of the extremities 5 days later. As illustrated in one rat (Fig. 1), lesions included degeneration of muscle fiber, disappearance of fiber, and a mild infiltration of round cells and polymorphonuclear leucocytes.

Viremia. Albino rats, other than suckling animals, exhibited minimal or no viremia. To determine whether viremia of greater degree might take place during the incubation period, 9 rats 26 days of age, were inoculated intraperitoneally. At 7 hours after inoculation and then daily for 6 days, blood was obtained from alternating pairs and pooled for testing. No virus was recovered from 1:10 suspensions of blood obtained from well rats during these periods, although 7 of the 9 eventually became paralyzed. Blood obtained from a pair with paralysis 3 days after inoculation had a virus titer of $10^{-2.7}$. Two other pairs of rats, however, which became ill 5 and 6 days after in-

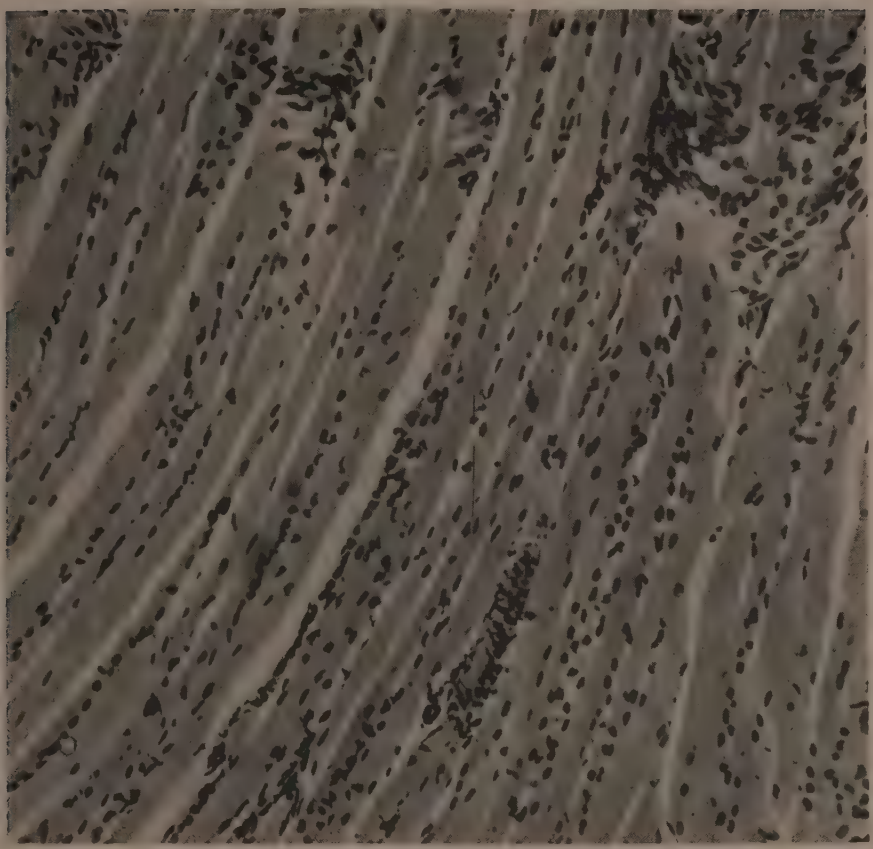

FIG. 1. Myositis in rat skeletal muscle (H & E × 170).

oculation, had no demonstrable viremia.

Inoculation of pregnant rats. Experiments on pregnant rats were performed to determine 1) if disease might be transmitted to offspring and 2) whether this virus, by growth in uterine muscle, might interrupt pregnancy. Seven rats, presumed to be pregnant, were inoculated intraperitoneally. Four of these gave birth to healthy litters 4 to 6 days after inoculation. Mothers and offspring remained well. Two rats, which proved not to be pregnant, developed paralysis of the hind legs, one in 7 days (weight 132 g) and one in 11 days (weight 236 g). Brains of both contained virus in high titer. The 7th rat gave birth in 3 days and at 7 days after inoculation she began dragging her hind-quarters but was still nursing. Of mice inoculated with a 1:10 brain suspension from this rat, only one of 3 died. Such a minimal amount of virus in an inoculated animal was regarded as being of questionable significance. No virus was recovered from a young one sacrificed a day after the mother became ill.

Ingestion and inhalation of virus. Albino and hooded rats were susceptible to infection both by ingestion and by inhalation. They are considered together, since no strain difference in susceptibility was ever apparent throughout present experiments. Of 18 rats 25 to 29 days of age inoculated intranasally with infected rat brain suspensions containing 10^{-3} to 10^{-6} LD_{50} of virus, 9 became paralyzed and died within 4 to 11 days. Two of the ill rats were tested for virus which, though readily recovered from other organs, was not

found in lung suspensions. Rats became ill less readily as a result of ingestion. Thus, 23 rats 3 to 4 weeks of age were fed infected carcasses of rats and mice. Of these, 2 were subsequently found dead and 2 developed paralysis, one in 6 days and another 2 weeks after feeding. EMC virus was recovered from brains of the ill rats. Seven of the survivors, tested for neutralizing antibodies 2½ to 3 weeks after an infective meal, were negative.

Comparative effect of other strains of EMC virus. A final question arose as to whether findings obtained in albino rats resulted from use of a particular strain of EMC virus, the one originally isolated from a sick baboon. For comparative purposes, the 2 other strains were inoculated into albino rats. These strains killed all rats under 2 weeks of age. Of 4 rats, 29-30 days of age, inoculation of the Florida strain intraperitoneally led to death of 2 and paralysis of another within 4 days. Inoculation of 3 rats of similar age with the rhesus strain led to paralysis of all in 5 days. Two rats were alive but crippled after 14 days. One acutely ill rat from both series was sacrificed. Patterns of virus distribution (rats 127W and 128W, Table I) did not differ essentially from those observed in albino rats inoculated with the baboon strain. Myocardium of rat 128W, however, had a titer of $10^{-2.5}$. Possibly this relatively high titer was related to the fact that the rhesus strain of EMC virus, as used in this experiment, had been previously passed through 7 intraperitoneal passages in multimammate rats(1).

Discussion. Induction of paralysis and death in albino rats is in contrast to reports of others(2-6) who described only inapparent infections. Although it is difficult, if not impossible, to explain differences in results, several factors should be considered. One is that only suckling rats are fully susceptible. A lesser number of older rats develop frank disease. As a rough estimate, based on overall experiments, 30% of rats inoculated intraperitoneally at 30 days of age and 10% of rats inoculated when 60 days or older might be expected to develop paralysis. These percentages would doubtless be increased by intracerebral inoculations. A second factor might be that some stocks of albino rats are resistant. Our results, however, were obtained with 3 different strains obtained in Africa and, in an additional experiment, non-inbred albino rats (Osborne-Mendel strain) from the National Institutes of Health, Bethesda, Md., proved susceptible to EMC virus. Strain of virus did not appear to be a factor of importance, since EMC virus originating from Dania, Florida, also led to death and paralysis. The widespread infection with viremia encountered in suckling rats and the limited distribution of virus in adults were findings somewhat parallel to those described by McLaren and Sanders (9). These investigators found that EMC virus grew well in muscle and brain of 5-day-old mice, but not as well in muscle af 30-day-old mice, although brains of the latter remained fully susceptible.

A question of importance is to what extent are titers the result (a) of intrinsic growth of virus within an organ and (b) of residual blood contained in the organ. If titers were secondary effects of a viremia, one would expect to observe 2 types of relationship. First, blood-containing organs such as the spleen should have relatively high titers and, second, any excised specimen of muscle or washed piece of ileum might be expected to have a titer at least 2 logs lower than that of the blood, if one may make estimates from blood volumes calculated by Kaliss and Pressman (10) for mouse organs in which blood vessels were tied off. As shown in Table I for suckling rats, virus presumably multiplied in all organs tested.

Summary. 1. EMC virus can produce a crippling and usually fatal illness in albino rats of all ages. 2. Suckling rats are more susceptible than older rats and experience a greater degree of systemic infection. 3. In older rats, the disease is chiefly localized in the central nervous system, although myositis was likewise observed.

1. Kilham, L., Masen, P., and Davies, J. N. P. Mss. in preparation.

2. Findlay, G. M., and Howard, E. M., *J. Path. Bact.*, 1951, v63, 435.

3. Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, v72, 407.

4. Powell, H. M., Jamieson, W. A., and Culbertson, C. G., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 80.
5. Schmidt, E. C. H., *Am. J. Path.*, 1948, v24, 97.
6. Warren, J., Russ, S. B., and Jeffries, H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 376.
7. Helwig, F. C., and Schmidt, E. C. H., *Science*, 1945, v102, 31.
8. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
9. McLaren, A., and Sanders, F. K., *Atti Del Vi Cong. Internazionale di Microbiol. Roma*, 1953, v3, 11.
10. Kaliss, N., and Pressman, D., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 16.

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Glucagon Content of Pancreatic Tissue Devoid of Alpha Cells.* (22042)

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(Introduced by G. L. Duff.)
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In spite of the large amount of work done in the past 30 years to determine the site of origin of glucagon, there is as yet no general agreement as to whether or not glucagon is the alpha cell hormone(1-5). Some have considered that the alpha cell is the source of glucagon because of differences in insulin requirement of alloxan(6) and metahypophyseal diabetes(7) as compared to that of surgical diabetes(8). This opinion is supported by the presence of a hyperglycemic factor in extracts from normal, alloxanized, and alloxanized duct ligated pancreas(3). An alternative explanation for the differences in insulin requirement was that in surgical diabetes intestinal absorption of nutriments is limited(9). This latter view was controverted by other experiments with duct ligated alloxan diabetic dogs(10). A hyperglycemic factor, apparently identical with glucagon, is present in the stomach of the dog. This was considered to confirm the origin of this substance from the alpha cell of the pancreas because of supposed similarity of argentaffin cell of the stomach with the alpha cell(1). Furthermore in one report, gastro-intestinal argentaffin cells are implicated in disturbances in carbohydrate metabolism(11). However, other workers have not found any correlation between tumours of these cells and diabetes(12). Similarly, glucagon could not be extracted from the stomach of the hog despite the presence of argentaffin cells(1). The lack of identity between the alpha cell and the intestinal argentaffin cell, originally demonstrated by Masson in 1924, has recently been confirmed(13, 14). It would therefore be erroneous to consider that the presence of a hyperglycemic factor in tissues containing argentaffin cells supports the conclusion that the alpha cells serve as the source of glucagon. The discovery of chemical agents apparently capable of damaging alpha cells, rather than clarifying this problem, gave further contradictory results(3-5). Some workers reported alleviation of diabetes by cobalt(15,16), and diethyldithio carbamate(16). Others reported no effect of either of these agents or of synthalin A on the severity of alloxan diabetes (13,17,18). In some studies extracts from the pancreas of cobalt treated animals have been reported to contain lesser quantities of glucagon than normal(4,19). On the other hand, one of us(5,20,21), using qualitative methods of glucagon assay could detect no significant differences between normal and cobalt treated pancreas. Others have reported that cobalt treatment increases the glucagon content of the rabbit pancreas, while synthalin A causes its complete disappearance(22). In this connection, it is of interest that the lesions reported by various investigators after cobalt, are not always identical; it seems to vary with the animal species, the dose and the route of

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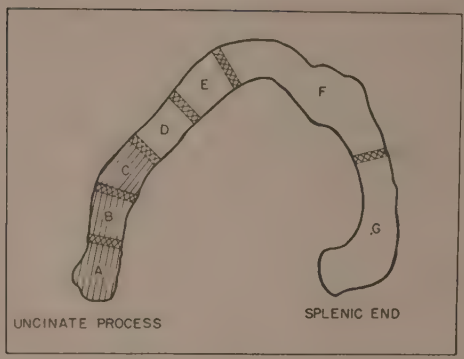

FIG. 1. Tracing of outline of dog pancreas. Longitudinally striated portions A, B and C were free of alpha cells while the remainder contained these cells. Cross hatched areas indicate tissue examined histologically.

administration(4,5,13,23,24).

As was previously pointed out, it has not been possible by the use of cobalt to prepare pancreas which are completely devoid of alpha cells(4,5). However, it has recently been demonstrated in this laboratory that the dog pancreas can be divided into two portions; one which does not contain alpha cells and which corresponds roughly to the uncinate process, and the balance of the pancreas which contains alpha cells(25). This finding enables a reinvestigation of the problem of the relationship of glucagon to alpha cells, since pancreatic tissue completely devoid of these cells is available for extraction.

Material and methods. The entire pancreas was removed from 6 normal mongrel dogs weighing between 15 and 20 kg which had been killed by exsanguination from the aorta after preliminary nembutal anesthesia. A tracing of the outline of the pancreas was made and the uncinate process was then divided into portions of approximately 1 to 2.5 g each. A complete transverse section of the proximal end of each portion was taken for histological examination and the position of these biopsies was recorded on an outline of the pancreas as indicated by the example in Fig. 1. In addition, transverse sections for morphologic study were obtained from the body and splenic end of the pancreas. The portions of the uncinate process and also the remainder of the pancreas were each immediately placed, separately, in acid alcohol and minced. Extracts prepared by the method of Best *et al.*(26), were dried and kept in the refrigerator until ready for use. The tissue for histological study was fixed in Zenker-Formol solution and sections were subsequently stained with Masson's trichrome, Gomori's chrome alum hematoxylin(27), Gomori's Aldehyde Fuchsin(28) and Mallory-Heidenhain-azan(29). In addition portions of the pancreas from the uncinate process and body

TABLE I. Blood Sugar Response of Fasting Anaesthetized Normal Cats to Intravenous Injection of Dog Pancreatic Tissue. (A) Containing alpha cells; (B) Devoid of alpha cells.

			Blood sugar, mg %					
				Min. after admin. of extract				
Material injected	Dose, g*	Dog	Before inj.	5′	10′	15′	20′	25′
A. Extract of alpha cell containing portion of pancreas	1	L 9	122	172	204	200		164
	"	"	94	131	142	147	133	109
	"	"	111	180	200	202	200	145
	"	L 12	115	169	202	197	187	177
	"	L 13	137	194	200	198	192	140
	"	"	124	137	200	200	182	182
	1/10	L 12	87	117	112	82	69	65
	"	"	102	126	124	110	107	92
	"	"	114	120	136	120	107	100
B. Extract of pancreatic tissue devoid of alpha cells (uncinate process)	1	L 9	112	110	108	100	85	67
	"	"	110	115	106		92	82
	"	"	104	100	92	83	74	66
	"	L 12	112	112	108	97	88	88
	"	L 13	129	127	120	112	101	103
	4	L 12	90	103	102	76	65	53
	"	L 15, 16, 17	114	120	119	94	90	82

* Calculated in term of wt of fresh pancreas.

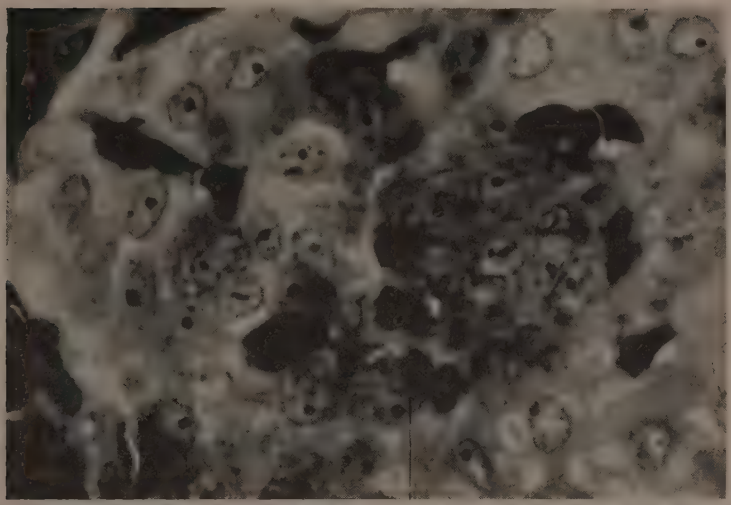

FIG. 2. Pancreatic islet from the alpha cell containing portion of pancreas. Dark finely granulated cells in center of islet are alpha cells and stained black in the original preparation. Zenker-Formol, Masson's Trichrome × 1045.

of 3 of the dogs were placed in 10% formol and impregnated with silver according to Fontana's method. For estimating the glucagon content the dried extracts were dissolved in physiologic saline (0.9% sod. chloride) acidified to pH 2.4 with HCl. One cc of solvent was used for each g of pancreas. The procedure suggested by Staub and Behrens(30) was followed. After an overnight fast, normal cats weighing between 2½ and 3½ kilo received 75 mg/kilo of sodium amytal. The inguinal veins were exposed bilaterally and duplicate blood samples were obtained just prior to, and at 5, 10, 15, 20, and 25 minutes after the injection of the test material into the contralateral vein. The blood glucose was determined by a micromodification of the Folin-Wu method(31). Extracts of 1 g of pancreatic tissue from each of the various portions were tested as per Table I. In addition 1/10 g portions of the body of the pancreas (Dog L 12) were tested in triplicate. Also, extract from 4 g of the uncinate process of Dog L 12 and a 4 g portion prepared by pooling the extracts from the uncinate process of dogs L 15, 16, 17, were used.

Results. Examination of the histological sections showed that most of the uncinate process, which amounts to approximately 15 to 20% of the total weight of the pancreas, is devoid of alpha cells. The islets of this portion are smaller than those of the rest of the pancreas and are roughly estimated to be, at most, only 1/10 of the volume of the latter. In addition, the islets of the uncinate process contain certain cells which stain red with the Masson trichrome in contrast to the alpha cells which stain black when a well ripened haematoxylin is used (Fig. 2 and 3). Argentaffin cells were demonstrated by the Fontana silver method in both portions of the pancreas, and seemed to be related to the medium sized ducts. No difference was observed in their number in either area.

The changes in blood sugar levels of the fasting anesthetized cats after the injection of each extract are represented in Table I. As was to be expected, the alpha cell containing portion of pancreas uniformly elicited an initial marked hyperglycemic response. The peak value, with 1 cc of extract, was reached at 10 to 15 minutes after injection and ranged from 53 to 91 mg% above the initial level. The blood sugar did not return to the fasting value by 25 minutes. On the other hand, one cc of extract from the portion of pancreas devoid of alpha cells did not cause an initial rise in the blood glucose concentration, except in

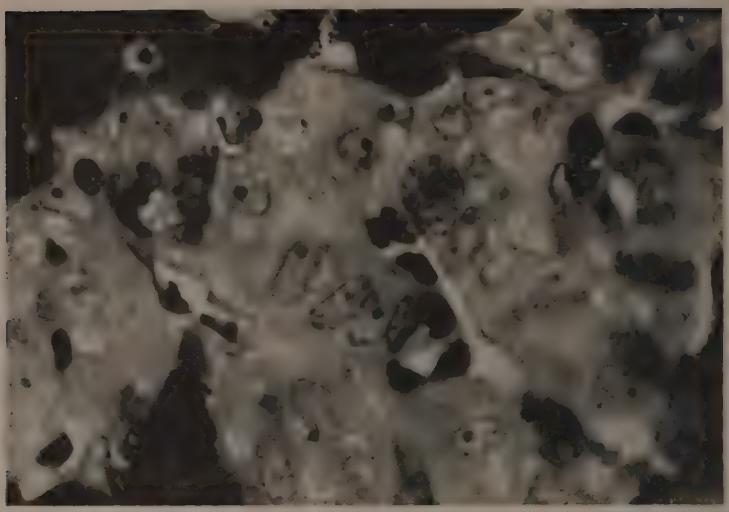

FIG. 3. Pancreatic islet from the region of pancreas devoid of alpha cells. Dark cells with rather coarse granules are X cells and stained deep red in the original preparation. Zenker-Formol, Masson's Trichrome × 1045.

one instance (L 9), where it was only 5 mg%. The 10-minute value was only 4 to 12 mg below the fasting level. By 15 minutes the insulin effect was somewhat more obvious with values of 12 mg% to 25 mg% and at 25 minutes the values varied from 24 to 45 mg% below the fasting level.

In view of the differences in islet volume observed morphologically 1/10 g equivalents of extracts from alpha cell containing portions and 4 g equivalents from the uncinate process which is devoid of alpha cells were also tested.

With the 1/10 g portions from the body, the initial hyperglycemia was less, ranging from 22 to 30 mg% above the fasting level and the peak was reached at 5 to 10 minutes after the injection. Thereafter, the blood sugar declined so that at 25 minutes the value ranged from 10 to 22 mg% below the initial level (Table I). The 4 g portion, from the areas devoid of alpha cells, also elicited a very mild initial blood sugar rise of 6 and 13 mg% above the fasting value at the 5 minutes specimen. This was followed by a decline of the blood sugar of 37 mg% and 32 mg% respectively at the 25 minutes specimens.

Discussion. The results of these experiments demonstrate an almost complete lack of hyperglycemic effect of extracts from the portion of pancreas devoid of alpha cells as compared to the remainder of the pancreas. However, the fact that a significant decline of the blood sugar did not occur until the 15 minutes specimen when 1 g portions of uncinate process were used, suggests the presence in the sample of an anti-insulin factor. The very mild initial hyperglycemia obtained with 4 g portions leads to a similar inference. Whether despite the lack of alpha cells this anti-insulin effect is due to glucagon, is open to question. Since the whole area of pancreas used for the extract cannot be examined in each case, it might be argued that some alpha cells were present. However, in other work it was demonstrated by consecutive longitudinal sections that the transition between the area devoid of alpha cells and the alpha cell containing area is quite abrupt(25). Therefore complete cross sections of the pancreas as performed in this study enable the separation of these two areas. It may be that the moderate hyperglycemia in this instance is a non-specific effect due to the large amount of foreign protein present in crude extracts.

Because of the interest in the argentaffin cells as a possible source of glucagon, these were also studied. However, only very occasional Fontana silver positive cells which

identify the argentaffin cells, were found randomly distributed throughout the entire pancreas. It is therefore most unlikely that these cells do serve as a source of glucagon.

In view of the blood sugar changes obtained with the extracts from the uncinate process, it is not possible to conclude definitively that glucagon is derived from alpha cells. However, the clear differences in the results when 1 g aliquots from 2 portions of the pancreas were used, does provide evidence for the view that alpha cells produce glucagon. At the present time, no other explanation is available for these differences.

Similar conclusions have been drawn from quantitative assays of extract of cobalt treated guinea pig pancreas which showed a 60% reduction in glucagon content(31). In another study using a qualitative method of assay, a mean diminution of 10% in the hyperglycemic activity of extract of cobalt treated dog pancreas, as compared with the normal was reported(19). On the other hand no significant difference in the glucagon content of extract of cobalt treated and normal dogs were found by other workers(5,20,21). These workers stated that since many alpha cells were still left in the pancreas after cobalt treatment, and since the method of assay was qualitative rather than quantitative, a moderate reduction in glucagon content may not have become apparent. In the present study although there was a marked reduction of glucagon content in extracts from the uncinate process, the blood sugar curves from this area still indicate a minimal anti-insulin effect. This may have been a non-specific action because of the crudity of the extracts. Further assays of purified material seem to be necessary to resolve the problem of whether small amounts of glucagon are actually present in the area devoid of alpha cells.

Summary. Most of the uncinate process of the dog pancreas is devoid of alpha cells, whereas these are found in the remainder. On the other hand argentaffin cells are sparsely but evenly distributed throughout the pancreas. Extracts of the portion of pancreas devoid of alpha cells show no hyperglycemic activity when 1 g aliquots are used. When 4 g portions are employed, a very mild initial hyperglycemia does occur. These findings are interpreted to support the opinion that glucagon originates from the alpha cells but are not definitive.

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1. Sutherland, E. W., *Recent Progress in Hormone Research,* 1950, v5, 441. Academic Press Inc. New York, N. Y.

2. Foa, P. P., *Advances in Internal Medicine* 1954, v6, 29, The Year Book Publishers Inc.

3. Cavallero, C., *Rev. Canad. de biol.,* 1953, v12, 509.

4. De Duve, C., *Lancet,* 1953, v2, 99.

5. Volk, B., Lazarus, S. S., and Goldner, M. G., *Arch. Int. Med.,* 1954, v93, 87.

6. Thorogood, E., and Zimmermann, B., *Endocrinology,* 1945, v37, 191.

7. Marks, H. P., and Young, F. G., *J. Endocrinol.,* 1939, v1, 470.

8. Gaston, E. A., *New England J. Med.,* 1948, v238, 395.

9. Mirsky, I. A., Futterman, P., Wachman, J. and Perisutti, C., *Endocrinology,* 1951, v49, 73.

10. R-Candela, J. L., Tamarit, J., R-Candela, R., and Cristensen-Konig, W., *Arch. Med. Exp.,* 1951, v19, 11.

11. Durieux, J., and Perruchio, P., *Biologie Medical,* 1953, v42, 562.

12. Weisberg, H. F., and Schaefer, G. L., *Am. J. Clin. Path.,* 1952, v22, 1169.

13. Fodden, J. H., *Am. J. Clin. Path.,* 1953, v23, 994.

14. Davis, J. S., *J. Path. and Bact.,* 1952, v64, 575.

15. Avezzu, G., and Luise, R., *Boll. Soc. ital. biol. sper.,* 1951, v27, 1515.

16. Mayer, J., Andrus, S. B., and Silides, D. J., *Endocrinology,* 1953, v53, 572.

17. Volk, B. W., Lazarus, S. S., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 406.

18. Lazarus, S. S., Goldner, M. G., and Volk, B. W., *Metabolism,* 1953, v11, 513.

19. Dorner, M., and Stahl, J., *Comp. rend. Soc. de biol.,* 1954, v148, 590.

20. Lazarus, S. S., Volk, B. W., and Goldner, M. G., *Metabolism,* 1954, v3, 449.

21. Goldner, M. G., Volk, B. W., and Lazarus, S. S., *J. Clin. Endocrinol.,* 1954, v14, 184.

22. Fodden, J. H., and Read, W. O., *Endocrinology,* 1954, v54, 303.

23. Van Campenhout, E., Cornelis, G., and Deulin, Th., *Ann. d'endocrinol.,* 1954, v15, 89.

24. Bencosme, S. A., personal observation.

25. Bencosme, S. A., and Liepa, E., *Endocrinology,* in press.

26. Best, C. H., Haist, R. E., and Ridout, J. H.,

J. Physiol., 1939, v97, 107.
27. Bencosme, S. A., *Arch. Path.*, 1952, v53, 87.
28. Gomori, G., *Am. J. Clin. Path.*, 1950, v20, 665.
29. Thomas, T., *Am. J. Anat.*, 1937, v62, 31.
30. Staub, S., and Behrens, O. K., *J. Clin. Invest.*, 1954, v33, 1629.
31. Hawk, P. B., Oser, B. L., and Sommerson, W. H., *Practical Physiological Chemistry*, 13th Edition 1954, Blakiston Co. Inc., Toronto.
32. Vuylsteke, C. A., Cornelis, G., and De Duve, S., *Arch. Internat. Physiol.*, 1952, v60, 128.

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Phagocytosis by HeLa Cells and Their Susceptibility to Infection by Human Tubercle Bacilli.* (22043)

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In order for an infectious agent to multiply within its host cell it must, of course, enter the cell by passing through the cell boundary. Since this portion of the cell is relatively impermeable even to molecules of moderate size, the passage of a particle as large as a bacterium or virus might be expected to be an unusual process. In the case of some of the bacteriophages it is apparently necessary only for the nucleic acid portion of the viral particle to enter the cell, the structural material of head and tail remaining on the outside of the cell wall(1). Such dissociation of viral nucleic acid from protein may later be found also in the animal viruses, yet it seems certain that with some of the larger infectious agents which multiply intracellularly, such as the rickettsiae and the leprosy bacillus, that the infectious agent enters the cell in intact form. The mechanism by which the infectious agent passes the cell membrane would thus appear to be an important factor in determining whether or not a cell is susceptible, that is, whether the agent can multiply within the cell. One such mechanism by which the infectious agent could pass the cell membrane could be the engulfment of the particle by the host cell, and it is possible that many of the differences between the growth of bacterial and animal viruses depend upon this activity by animal cells. The engulfment of droplets (pinocytosis) has been observed in "phagocytic" cells and cells of malignant origin, including the HeLa cell, by Gey *et al.*(2) and the engulfment of particles (phagocytosis) has also been described for many animal cells. The categoric separation of cells into "phagocytes" and "non-phagocytes" is unfortunate in this connection, because the difference between cells such as macrophages and fibroblasts is in degree of engulfing activity, rather than presence or absence of this activity.

Experiments described below deal with HeLa cells and the relationship between their phagocytic activity and the culture medium placed on them. It is seen that when HeLa cells are made more phagocytic by the use of media containing certain horse serums, human tubercle bacilli quickly enter the cell where they then grow luxuriantly, even when the medium is subsequently changed to one containing human serum, in which phagocytosis is at a minimum. If one examines a HeLa cell culture a few days after it was infected with the tubercle bacilli, large increases in the amount of intracellular growth are observed to result from the use of suitable horse serum media during the first day of infection, so that in effect the HeLa cells have been rendered more susceptible to this infection.

Materials and methods. The HeLa cells were grown on cover slips in flat-sided tubes. The cultures of the HeLa cells were started from cultures in 400-ml square bottles, the growth being washed 3 times with BSS (Hanks' balanced salt solution without sodium bicarbonate) then pushed off with a rubber policeman into 5 ml of the growth medium of

* The author wishes to acknowledge the capable technical assistance of Mrs. Mary Eleanor Jones.

Syverton, Scherer, and Elwood(3). Cell clumps were broken up with bulb and pipette and the suspension transferred to a flask containing the remainder of the growth medium, 50-70 ml per bottle. The cells in growth medium were then distributed in 1.0-ml amounts into the test tubes. After 2 days the cover slip was washed twice in the tube with 1.0 ml of BSS, 1.0 ml of the experimental medium was introduced, and 0.05 ml of the bacterial suspension added. The bacterial suspension was prepared from 4-7 day cultures of the H37Rv strain of the human tubercle bacillus grown in Dubos media containing 0.05% Tween, from which the bacilli were sedimented once in the centrifuge and resuspended in BSS by several forceful expulsions against the bottom of the tube with bulb and pipette. Phase microscopy showed the organisms to be present as individuals or small groups, and plate counts on 25% blood agar showed there were about 4 x 10^5 organisms (or clumps) in the 0.05 ml of inoculum introduced into the tissue cultures. At appropriate intervals after inoculation, coverslip cultures were prepared for microscopic study by washing twice in BSS, fixation in 10% neutral formalin, staining with carbol-fuchsin, destaining with 1% HCl in 95% ethanol, and counterstaining for 10 minutes with Giemsa. The media were changed in the remaining cultures after 2 days. All incubations were at 37°C. Suspensions of carbon were prepared from India ink according to Hanks(4). The experimental media used were variations of medium A, that is, 50% BSS and 40% serum, usually human or horse. The sera were all inactivated at 56°C for 30 minutes. Ten per cent chick embryo extract was also present in the media used in the present work, but the described effects on phagocytosis will occur in its absence.

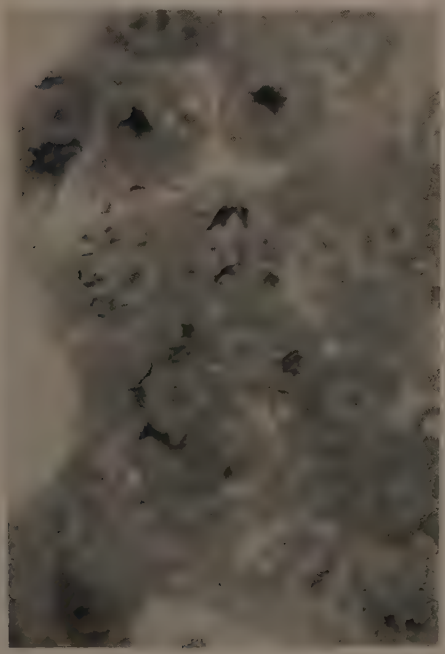

FIG. 1. HeLa cells, horse serum medium, one day after addition of tubercle bacilli. Many of the cells have taken bacilli into the cytoplasm.

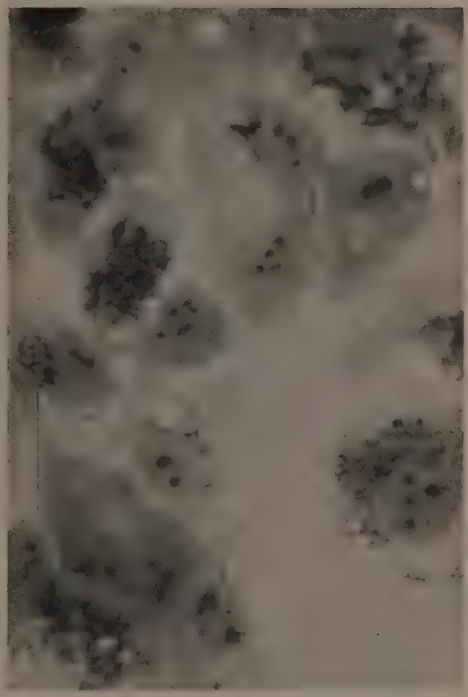

FIG. 2. HeLa cells, horse serum medium, 3 days after addition of tubercle bacilli. The bacilli in the cells have multiplied to fill much of the cytoplasm.

Results. Fig. 3 and 4 show the results obtained when the media contained human serum. Only a few cells (less than 1%) be-

FIG. 3. HeLa cells, human serum medium, one day after addition of tubercle bacilli. Only an occasional cell has taken in bacilli.

came infected. However, when the media contained a satisfactory horse serum, 50% or more of the cells became infected (Fig. 1). Tubercle bacilli within the cells then grew rapidly so that within 3-5 days large masses of bacilli were seen in the cytoplasm(Fig. 2). Smears of the culture fluid, which were done routinely, showed that although growth occurred in the fluid with the stated inoculum, no significant difference could be noted between the horse and human serum media.

The intracellular location of the organisms as seen in Fig. 1 and 2 was evidenced by the following observations. The bacilli, after a few days, were located around the nucleus with special concentration at the cytocentrum, as is characteristic of cytoplasmic inclusions. Washing the cells with BSS and changing to fresh media of the same constitution each day did not decrease the amount of intracellular growth. The inclusion of streptomycin in the culture media in concentrations of 4 γ/ml did not affect intracellular growth, although this concentration was enough to prevent extracellular growth. A change of the medium to one containing human serum did not alter bacillary growth within the cells. Lastly, sections of infected cells cut perpendicularly to the plane of growth showed the bacilli to be surrounded by cytoplasm.

That this effect was due to increased phagocytosis rather than to an increased invasive property of the bacilli as a result of growth in the tissue culture medium is indicated by experiments with nonliving particles. If the inoculum was a suspension of heat-killed tubercle bacilli or carbon particles, or both, these particles were found within the HeLa cells in much greater numbers with horse serum media than with human serum media (Fig. 5 and 6).

A number of other culture media have been tried but none has given as much phagocytic activity as the horse serum media described. Sera from other species, monkeys, rabbits, cattle, guinea pigs, and chickens, as well as bovine amniotic fluid, were also tried as substitutions for the serum in medium A. Although some samples of chicken serum and bovine amniotic fluid showed better results than human serum, none was as good as favorable samples of horse serum. Ultrafiltrates of horse, cow, rabbit, and human serum and bovine amniotic fluid were intermediate in effect and indistinguishable from each other. Not all samples of horse serum were effective and, despite extensive trials, we have not been able to predict without preliminary trial whether a certain lot will be suitable. However, repeated tests on a given lot have given entirely

FIG. 4. HeLa cells, human serum medium, 3 days after addition of tubercle bacilli. Not much change from day 2 (Fig. 3), although it is possible to find cells containing considerable bacterial growth.

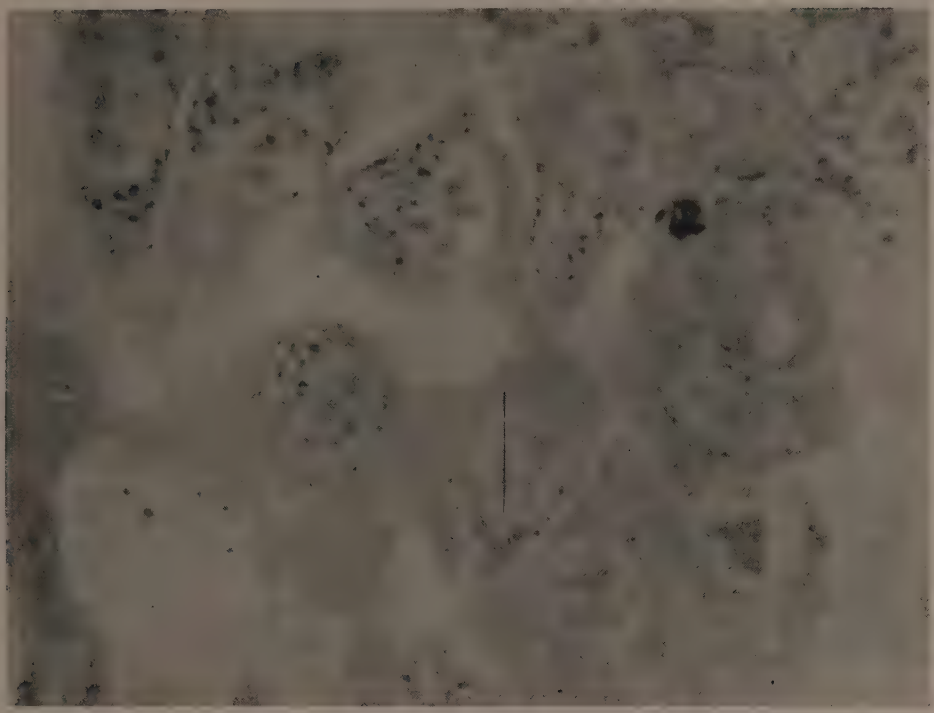

FIG. 5. HeLa cells, horse serum medium, one day after addition of carbon particles and dead tubercle bacilli. Many of the cells have taken the particles and bacilli into the cytoplasm.

uniform results over periods of at least 4-5 months.

Monkey kidney cell cultures in monolayers have shown essentially the same results, that is, favorable horse serum medium is able to increase phagocytosis of living and dead human tubercle bacilli and carbon particles. Luxuriant intracellular growth of the living tubercle bacilli then followed. The results in plasma clot cultures of human foreskin were not so easy to evaluate, perhaps because of the interposition of the clot between cells and culture medium.

The sensitivity of HeLa cell cultures in suitable horse serum culture fluid, as growth medium for small numbers of tubercle bacilli was indicated by several comparisons with bacteriological media, HeLa cell cultures detecting about as many bacilli (H37Rv) as Dubos medium with or without Tween (Difco), and about one-tenth as many as 25% human blood agar(5). It is interesting that the result in HeLa cells could be read in 3-5 days.

Because we were interested primarily in factors affecting intracellular bacillary growth (as a part of a study of tissue culture procedures which might be useful in a study of the leprosy bacillus), we have not attempted to develop methods for the accurate estimation of extracellular bacilli. The number of extracellular bacilli was studied only by smears of the culture fluid, and the strong tendency of the bacilli to clump subjects this method of estimation to considerable error. From the data obtained it is not possible to know whether the total amount of bacillary growth occurring in a culture tube, intra- and extracellularly, was increased as a result of the increased phagocytosis.

Discussion. Certain difficulties are met in interpreting results obtained in tissue culture according to the concepts of infectious processes which are derived mainly from the study of infections in intact animals. Thus it

FIG. 6. HeLa cells, human serum medium, one day after addition of carbon particles and dead tubercle bacilli. The intake is considerably less than that in horse serum (Fig. 5), although in the original preparations some small carbon particles may be seen in the cytoplasm of many cells.

is said that a certain cell or tissue culture is susceptible or resistant to a particular infectious agent, because of the practice of considering a mouse or guinea pig as being susceptible or resistant to a given agent. Obviously a more complete statement of experimental conditions is desirable with tissue cultures because of the greater possibility of affecting the multiplication of the infectious agent by changes in culture media, temperature, pH, conditions of inoculation, etc. Even the term "susceptibility" tends to become ambiguous, because in this study, for example, it appears that HeLa cells in our medium are resistant to human tubercle bacilli, yet after the bacilli have gained access to the cytoplasm as a result of use of horse serum media they grow very well in HeLa cells in 40% human serum. A further understanding of the processes by which phagocytosis occurs in a cell such as the HeLa cell may emphasize other semantic obstacles.

Summary. When human tubercle bacilli are added to cultures of HeLa cells, the amount of intracellular growth that ensues depends upon the culture medium on the HeLa cells. If the medium is one containing 40% human serum, 10% chick embryo extract, and 50% BSS, little growth is seen within cells. However, if instead of human serum, certain horse serums are used, tubercle bacilli are rapidly taken up by the cells and luxuriant intracellular growth follows, even if the culture medium is later changed to one containing human serum in place of the horse serum. The mode of action of the horse serum media appears to be due to an increase in phagocytosis, since such media also increase the amount of phagocytosis of dead tubercle bacilli and carbon particles.

1. Hershey, A. D., and Chase, M., *J. Gen. Physiol.*, 1952, v36, 39.

2. Gey, G. O., Shapras, P., and Borysko, E., *Annals N. Y. Acad. Sci.*, 1954, v58, 1089.

3. Syverton, J. T., Scherer, W. F., and Elwood, P. M., *J. Lab. Clin. Med.*, 1954, v43, 286.

4. Hanks, J. H., *Internat. J. Leprosy*, 1947, v15, 48.

5. Tarshis, M. S., Kinsella, P. C., and Parker, M. V., *J. Bact.*, 1953, v66, 448.

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Eosinophil Rhythm in Splenectomized Mice.* (22044)

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As an extension of related work on rats(1), the possible effects of splenectomy upon eosinophil counts and especially upon the 24-hour eosinophil rhythm of mice(2-5) were studied. Twenty-three B_1 males, 2½ months of age, were splenectomized, using Nembutal for anesthesia, while 22 comparable mice were subjected to a sham-operation. Purina Fox Chow and tap water were available to these mice *ad libitum* since weaning and throughout the experiment. For at least 7 days prior to experiment, the mice were singly housed and kept in a room illuminated by artificial light only. A clock-controlled switch turned the lights on at 06:00, and off at 18:00. In order to eliminate effects of previous handling upon the circulating eosinophil level, each group of mice was divided into 2 sub-groups. One subgroup from both the splenectomized group and the sham-operated group was used for sampling of tail blood at one of the 2 times of day chosen for study, namely between 07:00 and 08:30, and between 21:00 and 22:30. The methods employed for eosinophil counts have been described(6).

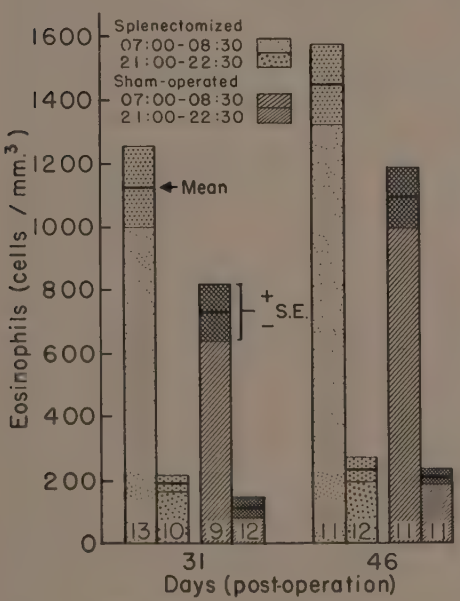

FIG. 1. Eosinophil levels of splenectomized and sham-operated B_1 male mice, at 2 times of day, 31 and 46 days after operation.

* This work was aided by grants from the Elsa U. Pardee Foundation, U. S. Public Health Service, Am. Cancer Soc. (Institutional and Minn. Division Grants), and Graduate School, University of Minnesota.

TABLE I. Comparison of Eosinophil Levels in Splenectomized Mice and in Sham-Operated Mice, at Several Post-Operative Time Points and at Several Times of Day.

Days after operation	Time of day	Diff. in mean eosinophil count*	t	P
31	7- 8:30	+399	2.41	.026
	21-22:30	+ 87	2.49	.022
46	7- 8:30	+361	2.18	.04
	21-22:30	+ 19	.42	>.60
68	2:50- 4:45	+285	3.12	<.01

* Mean eosinophil count of splenectomized group *minus* the corresponding value of sham-operated group.

The *results*, summarized in Fig. 1, reveal that at 31 and at 46 days after operation, the morning counts were consistently higher than the corresponding evening counts for both the splenectomized group and the sham-operated group ($P<0.001$ in all instances). Clearly, in mice as well as in rats, the spleen may not be necessary for the maintenance of the eosinophil rhythm.

A comparison of counts made at the same time of day in sham-operated mice and splenectomized mice (Fig. 1) also suggests the occurrence of a post-splenectomy eosinophilia, a phenomenon analyzed in Table I. This table includes additional data obtained 68 days after operation, and reveals that the magnitude of post-splenectomy eosinophilia is to some extent a function of the time of day at which the counts were done. But, at the daily times of high eosinophil levels (07:00-08:30),

the eosinophilia occurring during the second month after splenectomy in mice stands out with unmasked clarity and is statistically significant.

Summary. At several post-operative time points, eosinophil counts were made on tail blood from splenectomized and from sham-operated male B_1 mice, kept under standardized environmental conditions. The occurrence of post-splenectomy eosinophilia was noted. Furthermore, the mean eosinophil counts of all of the groups of mice studied were consistently higher by day than by night. In B_1 mice and rats the presence of the spleen is not essential for the maintenance of eosinophil rhythm.

1. Halberg, F., Albrecht, P., and Zander, H. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 404.
2. Halberg, F., and Visscher, M. B., *ibid.*, 1950, v75, 846.
3. Halberg, F., Visscher, M. B., and Bittner, J. J., *Am. J. Physiol.*, 1953, v174, 313.
4. Louch, C., Meyer, R. K., and Emlen, J. T., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 668.
5. Panzenhagen, H., and Speirs, R., *Blood*, 1953, v8, 536.
6. Halberg, F., Bittner, J. J., and Visscher, M. B., *ibid.*, 1951, v6, 832.

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Methemoglobinemia Induced by X-Irradiation. (22045)

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Oxidation reactions in aqueous media are commonly enhanced by exposure to ionizing radiation. The iron of the circulating hemoglobin, both reduced hemoglobin and oxyhemoglobin, is in the ferrous or reduced state. Ferric or oxidized hemoglobin occurs in methemoglobin, which amounts normally to 1 to 2% of the total blood pigment(1,2). Fricke and Peterson(3) found that oxyhemoglobin in solution disappears when it is irradiated. They concluded, from spectroscopic observations, that methemoglobin is the principal reaction product. This reaction has been studied to ascertain its usefulness as a quantitative biologic indicator of radiation exposure.

Normal human values. Methemoglobin was determined at various times in 8 apparently healthy laboratory workers, 7 male and one female, by the method of Evelyn and Malloy (4). Total blood pigment was determined after its conversion to methemoglobin by potassium ferricyanide.

The results are summarized in Table I. In our hands, this method was reproducible to ± 0.04% methemoglobin. The mean value of 33 determinations was 1.53 ± 0.30%. The normal methemoglobin level in a given individual was found to vary from time to time; the mean standard deviation of individual variations was 0.33% methemoglobin.

In vitro studies. Hemolyzed washed human red blood cells were mixed with M/60 phosphate buffer, pH = 6.5, in the proportions 1:1, 1:10, 1:20, 1:40, and irradiated with 260 kvp x-rays filtered by 1.0 mm Al and 0.5 mm Cu. The results of a typical experiment are shown in Fig. 1. For doses below 20000 r, the formation of methemoglobin was found to be a linear function of dose. Methemo-

TABLE I. Normal Levels of Methemoglobin (%).

E. R.	J. W.	J. T.	L. W.	W. K.	R. D.	G. S.	S. G.
1.53	.57 2.03 2.06	2.18 1.53	1.77	.53	1.29	1.93	.93
1.62	1.60 2.83 2.74	.82 2.50	1.58	1.82	1.03	1.70	1.23
1.92	1.14 .78 .86	1.06 1.23	2.32	.84	1.22	2.01	1.66
1.69 ± .17	1.66 ± .78	1.60 ± .66	1.89 ± .31	1.06 ± .55	1.18 ± .11	1.88 ± .13	1.27 ± .30

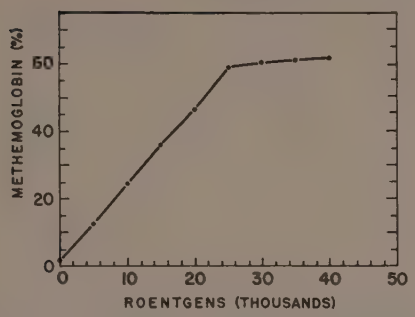

FIG. 1. Methemoglobin formed by irradiation of lysed washed RBC 1:40 in M/60 phosphate buffer, pH = 6.5.

globin was formed in the more dilute solutions at a relatively greater rate than would be expected from the concentration differences.

In vivo experiments. Ten adult male Sprague-Dawley rats were irradiated with 260 kvp x-rays filtered by 1.0 mm Al and 0.5 mm Cu at a rate of 50 r/min. Samples of tail blood for methemoglobin determinations were obtained pre-irradiation, during irradiation

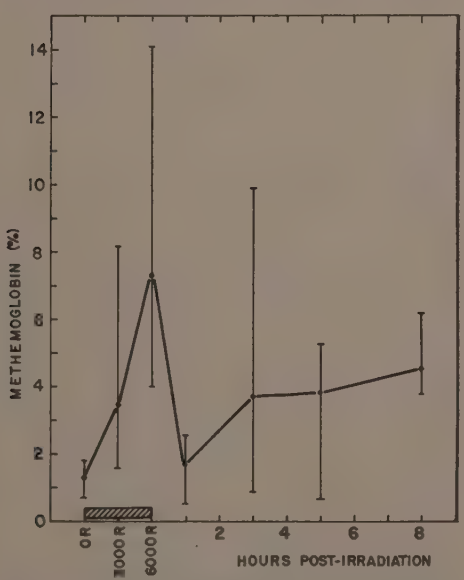

FIG. 2. Methemoglobinemia induced by 260 kvp x-rays in Sprague-Dawley rats with observations in the postirradiation period. Range of values and means are shown.

after 3000 r, and 6000 r and postirradiation after 1, 3, 5, and 8 hours. The results are depicted in Fig. 2. Variations in the normal methemoglobin values similar to the variations in the human determinations were observed.

Discussion. The oxidation of the ferrous iron within the hemoglobin moiety is presumably analogous to the radiation induced oxidation of ferrous salts in aqueous solution. The latter reaction is the basis of one kind of chemical radiation dosimeter(5). The reaction is probably mediated by the oxidative free radicals which are formed upon irradiation of water. In the case of inorganic iron salts, pH and substrate concentration affect the reaction rate. These parameters are held within narrow limits in the circulating blood.

The low one-hour postirradiation methemoglobin level is less than might be expected from reconversion of the methemoglobin in drug induced methemoglobinemias. In experimental drug induced methemoglobinemias the rate of reconversion to hemoglobin was found to be 8-10% per hour(6,7).

Although the increase in methemoglobin was observed to be proportional to radiation exposure in both the intact animal and *in vitro* studies for moderate doses, the usefulness of methemoglobinemia as a quantitative biologic radiation dosimeter appears to have several limitations. Variation was found in the normal baseline levels from individual to individual, and in a given individual from time to time. The secondary postirradiation methemoglobinemia found in the intact animals is a complication. Appreciable methemoglobinemia is produced only after exposures in the lethal and supralethal ranges.

Summary. Methemoglobin forms in aqueous solutions of hemoglobin and in intact rats after exposure to ionizing radiation. For moderate doses, the degree of methemoglobinemia is a linear function of dose. In the sub-lethal dose range, the methemoglobinemia is comparable to the random variation in normal values. A secondary methemoglobinemia was observed in rats 3 to 8 hours postirradiation.

1. Ramsay, W. N. M., *Biochem. J.*, 1944, v48, 470.
2. Van Slyke, D. D., Hiller, A., Weisiger, J. R.,

and Cruz, W. O., *J. Biol. Chem.*, 1946, v166, 121.
3. Fricke, H., and Petersen, B. W., *Am. J. Roentgen.*, 1927, v17, 611.
4. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, v126, 655.
5. Weiss, J., *Nucleonics*, 1952, v10, July 28.
6. Gelinsky, G., *Arch. f. exp. Path. u Pharmakol*, 1940, v195, 460.
7. Keise, M., *ibid.*, 1947, v204, 288.

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Relation of Sex and Age to Resistance of Mice to Experimental Histoplasma Infections.* (22046)

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Preliminary studies from this laboratory indicated that age affected the resistance of white Swiss male mice to experimental histoplasmosis(1). Campbell and Saslaw(2) using 14- to 17-g white mice noted a greater mortality in males. Salvin(3) reported no sex differences in mortality of 35-day-old mice. The purpose of this report is to elucidate further the sex and maturity factors relative to histoplasma infections in mice.

Materials and methods. Five-week-old white Swiss mice of both sexes were obtained at one- or 2-week intervals and kept until there were groups representing these age intervals from 5 through 23 weeks. The numbers of mice used in each group varied according to availability. The mice were inoculated intraperitoneally with 23 or 35 million yeast phase *H. capsulatum* organisms in 0.5 ml of 5% hog gastric mucin. All mice were weighed on the day of inoculation, and at weekly intervals. The mice were observed for a 30-day period and necropsy performed on those dying during this interval.

Results. Table I summarizes the results of these studies. Column A in Table I demonstrates that 10- to 15-week-old male mice receiving 23 million organisms showed increased resistance to fatal infection as compared to those 5 and 9 weeks old. A further increase in resistance was noted in those 16 weeks or older. With the 35 million dose (column C) the increased resistance was first noted at 17 weeks of age. The parallel studies in female mice with the lower dosage (column B) showed a similar increase in resistance at 8 weeks as compared to 10 weeks in males and a further increase in survival rate after 15 to 16 weeks of age. With the 35 million dose (column D) the 2 periods of comparative increased resistance were noted at 9 and 17 weeks in females and males, respectively. In comparing the death rates in both sexes receiving 23 million organisms the 8-week-old male mice showed 19 of 22 deaths (86.5%) as compared to 14 of 25 female deaths (56%); in the 9-week-old group, 9 of 10 males (90%), and 7 of 11 females (63.3%) died. Thereafter the incidence was similar except at 15 weeks when 8 of 12 males, as compared to 4 of 10 females, died. In those mice receiving 35 million organisms the differences in resistance of sexes became apparent in the 9-week-old mice where 15 of 16 males (93.7%) as compared to only 9 of 15 females (60%) died. This difference became less apparent by the 13th week and no appreciable variation was noted thereafter. Combining the total mortalities under both sex groups the 8- to 14-week period represented the time interval when apparent sex differences in susceptibility occurred (Table II). As can be seen in Table II, when all of the age groups were combined no significant difference between sexes was noted following inoculation with 23 million organisms, since 174 of 337 females (51.6%) died as compared to 183 of 331 males (55.3%). However, with the 35 million dose, the males were significantly more susceptible in that 105 of 145 males (72.5%)

* This investigation was supported in part by research grant from the National Institute of Health.

TABLE I. Effect of Age on Resistance of White Swiss Mice to Histoplasmosis.

Challenge dose, 23 million — Deaths					Challenge dose, 35 million — Deaths				
	A ♂		B ♀			C ♂		D ♀	
Age (wk)	No.	%	No.	%	Age (wk)	No.	%	No.	%
5	10/10*	100.0	10/10	100.0	5	15/15	100.0	15/15	100.0
6	31/32	96.8	32/33	96.9					
7	10/10	100.0	10/10	100.0	7	15/15	100.0	14/15	93.3
8	19/22	86.4	14/25	56.0					
9	9/10	90.0	7/11	63.3	9	15/16	93.7	9/15	60.0
10	13/25	52.0	13/26	50.0					
11	6/10	60.0	6/10	60.0	11	13/15	86.6	10/15	66.7
12	13/24	54.2	13/27	48.1					
13	8/11	72.7	6/10	60.0	13	10/12	80.0	11/16	68.7
14	16/26	61.5	14/23	60.8					
15	8/12	66.7	4/10	40.0	15	12/15	80.0	11/15	73.3
16	8/23	34.8	8/25	32.0					
17	3/9	33.3	4/10	40.0	17	7/15	46.7	6/15	40.0
18	6/27	22.2	8/26	30.7					
19	3/10	30.0	4/9	44.0	19	5/14	35.7	4/14	28.0
20	5/24	20.8	7/25	28.0					
21	3/10	30.0	3/10	30.0	21	7/15	46.7	5/14	35.7
22	8/26	30.7	7/27	25.9					
23	4/10	40.0	4/10	40.0	23	6/14	42.8	7/15	46.7

* Numerator = No. of deaths. Denominator = Total No. inoculated.

died as compared to 92 of 149 females (61.7%). As can be seen in Table III, the increase in resistance was not merely a difference of weight. For instance, while the weight of male animals from 5-9 weeks of age increased 4.4 g (12.9 to 17.3 g) there was no significant increase in resistance, but between the 9th and 10th week the fatality dropped from 94.4 to 59.2% while the weight increased only 0.2 g.

Discussion. The data presented in these studies suggest that age, sex and dosage affect the susceptibility of white Swiss mice to experimental histoplasma infections. Increased resistance became apparent by the 8th and 10th weeks, respectively, in females and males when 23 million organisms were inoculated. With more severe challenge (35 million organisms) increased resistance was noted at 9 weeks of age in females; the males showed equal susceptibility through 15 weeks, but increased resistance at 17 weeks. These latter results differ slightly from those previously reported by us in which the period of increased resistance in males was noted at 14 weeks of age(1). This difference may be attributed to the fact that a more careful definition of age was made in this study in that all mice were originally obtained at 5 weeks of age, and then held until the desired age group was obtained. In the previous study the specified age groups were ordered from the animal farm which could have resulted in more variation in comparative ages. Thus, it appears that females become more resistant at a slightly earlier age (8 weeks, as compared to 10 in males) with 23 million organisms as inoculum, but this difference was more marked after the 35 million dose, in that females showed significantly greater survival as early as 9 weeks of age, as

TABLE II. Comparison of Resistance of Male and Female Mice of Same Age Groups.

Dose (million)	Sex	5-7-wk mice — Deaths No.	%	8-14-wk mice No.	%	15-23-wk mice No.	%	Total No.	%
23	♀	52/53*	98.1	73/132	55.3	49/152	32.2	174/337	51.6
23	♂	51/52	98.0	84/128	65.6	48/151	31.7	183/331	55.3
35	♀	29/30	96.7	30/ 46	65.2	33/ 73	45.2	92/149	61.7
35	♂	30/30	100	38/ 43	88.4	32/ 72	51.4	105/145	72.5

* No. of deaths/No. of mice infected.

TABLE III. Comparison of Weights with Age and Resistance of Mice.*

Age (wk)	Sex	Variations in avg wt (g) of age groups	Increase in avg wt (g) of age groups	Avg % fatalities of group
5- 9	♂	12.9-17.3	4.4	94.4
10		17.3-17.5	.2	52.0
11-15		17.5-21.3	3.8	59.2
16		21.3-21.4	.1	34.8
17-23		21.4-23.5	2.1	28.7
5- 7	♀	12.6-15.3	2.7	98.1
8		15.3-15.7	.4	56.0
9-14		15.7-19.0	3.3	55.3
15		19.0-19.3	.3	40.0
16-23		19.3-22.4	3.1	32.2

* Inoculated with 23 million yeast phase *H. capsulatum* in 0.5 ml mucin.

compared to 17 weeks in males. Between 8 to 14 weeks of age females appeared to be especially more resistant than males. This observation can probably be attributed to the fact that female mice mature more rapidly than males(4). Thus, before and after full development of sexual maturity the differences in susceptibility were not appreciable. These results are also comparable to those observed by Theiler(5) in which the morbidity and mortality rates in experimental encephalomyelitis of mice were lower in mice over 12 weeks of age. The recognition of such differences in susceptibility in different age and sex groups can assist in the evaluation of data in a variety of studies employing the mouse as the experimental animal.

Conclusions. Age, sex, and dose are important factors in comparative susceptibility of mice to fatal histoplasma infections. Both sexes may show lower mortality ratios with increasing age. Females develop resistance earlier than males. This difference is most apparent between 8 to 14 weeks.

1. Schaefer, J., and Saslaw, S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 223.

2. Campbell, C. C., and Saslaw, S., *Pub. Health Rep.*, 1951, v66, 570.

3. Salvin, S. B., *J. Immunol.*, 1953, v70, 267.

4. Snell, G. D., in *Biology of the Laboratory Mouse*, 1941, p59, Roscoe B. Jackson Memorial Laboratory, Blakiston, Phila.

5. Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, v49, 67.

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Laboratory and Clinical Observations on Mecamylamine as a Hypotensive Agent.* (22047)

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Currently available ganglionic blocking agents used for the treatment of hypertension produce an inconstant day to day reduction in blood pressure largely due to incomplete and variable absorption of the quaternary ammonium compounds (bisammonium) from the intestinal tract. Mecamylamine, a secondary amine† is the first known ganglionic blocking agent which is completely absorbed after oral administration. The hemodynamic effects have been studied both in the laboratory as well as in patients with hypertension.

Methods and materials. Laboratory observations: Observations on the blood pressure response and on ganglionic blockade were made on 29 dogs anesthetized with 30 mg/kg of pentobarbital. The dogs were divided into 2 groups. Group I consisted of 12 dogs on which observations were made relative to doses which effectively blocked the autonomic ganglia and consequently reduced the blood pressure. Group II consisted of 17 dogs on which observations on the effect on renal hemodynamics and the excretion rates of water and electrolytes were made. *Group I.* Arterial blood pressure was measured by direct intra-arterial manometry in these ani-

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† 3-Methylaminoisocamphane hydrochloride. Available as Inversine by Sharp & Dome, Division of Merck & Co., West Point, Pa.

mals. Following suitable control observations, dosage increments of .05, .5, 1 and 2 mg/kg of mecamylamine were given intravenously over a 5-minute period. Subsequently, observations on blood pressure were made for the ensuing 5 hours. During this period, the blood pressure responses to electrical stimulation of the peripheral end of the severed vagus and to carotid occlusion were made at regular intervals. At one hour intervals, norepinephrine was infused at the same rate as required to produce a rise in mean blood pressure of 20 mm Hg during the control period. This was done as a test for adrenergic blockade. *Group II.* (*Renal studies*). Seventeen female dogs ranging in weight from 9 to 15 kg were used. They were anesthetized with pentobarbital, 30 mg/kg body weight, given intravenously. Creatinine was used to measure glomerular filtration rate (GFR) and p-aminohippurate (PAH) for renal plasma flow (RPF). Sodium and potassium determinations were made using a Beckman flame photometer for analysis. Arterial blood, collected in heparinized syringes, was used for chemical analysis. Analytical methods and procedures have been described previously(1). Mean blood pressure (MBP) was measured by a damped mercury manometer connected through a manifold to an indwelling arterial needle.

Clinical observations. The clinical observations were made on 2 groups of patients with moderately severe to severe hypertension. Group I consisted of 12 patients who were observed in detail and in whom observations were made on single dosage responses giving the drug no more frequently than once every other day. Group II consisted of 50 patients who received the mecamylamine as therapy for their hypertension. *Group I.* The 12 patients in Group I consisted of hospitalized patients with moderate to severe hypertension. After suitable control observations were made on the day of the test, 5 mg of mecamylamine were given orally at 8 o'clock in the morning. Observations on blood pressure were again made in the supine and upright positions every 15 minutes for the first 3 hours and then every half hour for the remainder of the day. If an adequate reduction in blood pressure was not obtained, a larger dose was given 2 or more days later and the procedure repeated. The dose was thus increased in 5 mg increments until an effective reduction in blood pressure‡ was obtained for each patient. Subsequently, 5 of the patients were given the same dose (effective dose) by the parenteral route in order to compare the response by the 2 different routes of administration. *Group II.* (Out-patients with Hypertension): All patients in this group had received 1 mg of reserpine a day for several months before initiation of the current study. This was not altered. None of the patients had become normotensive and control observations for the current study were made 2 months or more after stabilization on reserpine. The reserpine was continued because it reduces the pulse rate and blocks the reflex orthostatic tachycardia which is frequently observed with patients given ganglionic blocking agents.

This study consisted of a group of 50 patients with moderate to severe essential hypertension treated as out-patients in the Hypertensive Clinic. All patients had control blood pressures, as measured in both the supine and upright positions, of 150/100 mm Hg or greater. Controls were established by taking the average of a minimum of 4 separate blood pressure and pulse rate determinations in the supine and upright positions while the patients received only placebo. The post drug observations (2 months or more) are the averages of all blood pressures and pulse rates taken weekly in both the supine and upright positions after the therapeutic dose of mecamylamine for each patient had been established (*i.e.*, after the titration period during which time blood pressure and drug dosage were being stabilized). Sixteen of the group were males and 34 were females. Five were white and 45 negro. Average age was 52 years, with a range of 34 to 69 years. The severity of the disease in this group is best indicated by a high incidence of complications and by the fact that 41 (82%) had the retinal

‡ An attempt was made to reduce the standing blood pressure to a level below 150/100 without prohibitive side reactions.

TABLE I. Mean Blood Pressure* Response to Graded Doses of Mecamylamine Given Intravenously to Dogs.

Dog No.	Control	Time after drug in min. 5	10	15	20	30	45	90	120	300	Dose, mg/kg
1	128	96	90	84	80	60	55	50	44	44	2
2	132	110	100	106	104	100	102	100	102	104	2
3	142	98	100	104	116	102	104	110	128	136	2
4	164	154	144	128	126	128	134	142	146	146	.5
5	115	108	106	100	90	84	70	65	70	88	.5
6	168	142	135	136	138	132	128	128	126	130	.5
7	140	132	128	116	114	96	94	94	112	104	1
8	128	120	100	85	82	80	90	80	95	95	1
9	110	65	65	60	50	54	60	58	76	88	1
10	138	140	140	138	130	134	130	126	128	128	.05
11	138	136	138	138	140	140	144	146	148	154	.05
12	160	160	158	156	164	160	162	158	160	160	.05

* Direct intra-arterial manometry.

changes of hypertensive vascular disease; 7 of these had retinal hemorrhages. The initial dose of mecamylamine was 5 mg given orally twice a day (after breakfast and supper). This dose was empirically chosen as one which would not produce excessive hypotension or side reactions. However, in some cases, this dose resulted in marked orthostatic hypotension and numerous side reactions. A more suitable starting dose would be 2.5 mg twice a day. In our experience, this dose rarely produces side reactions or excessive hypotension. The initial dose was gradually increased in a stepwise fashion until an adequate blood pressure response was obtained. To minimize the orthostatic side reactions from excessive hypotension, the blood pressures in the upright position were the guide to the weekly changes in dosage. In addition to the blood pressure observations, clinical manifestations of excessive hypotension such as dizziness on standing and weakness were considered when regulating the dosage schedule.

Results. General Laboratory Observations (*Group I*): A consistent blood pressure effect in dogs was not observed with doses of less than 0.1 mg/kg. Doses in excess of 0.5 mg/kg did not cause much greater reduction in blood pressure than the 0.5 mg/kg dose but the period of the depressor response was not as long with smaller doses as with the larger ones. Likewise, the onset of action was somewhat more rapid with larger doses. Following administration of 0.5 mg/kg or more, this delay period was 5 to 10 minutes before the blood pressure decreased, and maximum depression was observed after 30 to 45 minutes (Table I). Blockade of the depressor response to vagal stimulation was consistent at the 1 mg/kg level. Blockade of the carotid pressor reflex usually occurred at a dose of 0.5 mg/kg. When maximum reduction in blood pressure occurred, there was no evidence of adrenergic blockade. In fact, the dose of norepinephrine required to produce a rise in mean blood pressure of 20 mm Hg during the control period was usually excessive after the administration of mecamylamine, apparently a result of ganglionic blockade of vasopressor inhibitory reflexes which are mediated via the parasympathetics. These observations indicate that the vasodepressor effect is not due to adrenergic blockade. Ganglionic blockade (partial) is present, but an additional central component has not been ruled out.

Observations on Renal Hemodynamics (Group II): Doses of mecamylamine of 0.2 and 0.5 mg (approximately .03 to .05 mg/kg) had no effect on blood pressure, renal hemodynamics, or on water and electrolyte excretion. Doses of 0.5 to 2.0 mg/kg produced a significant reduction in blood pressure ($p < 0.001$) which was more prolonged after the 1 and 2 mg/kg doses than after the .5 mg/kg dose. However, the initial reduction in blood pressure appeared to be nearly the maximum response with the 0.5 mg dose and was not increased by giving a larger dose. The initial reduction in blood pressure was not associated with depression in glomerular filtration rate or in renal blood flow during the first 30 minutes after drug administration. Renal vasodilata-

TABLE IIA. Effect of Mecamylamine on Renal Hemodynamics.

Dog No.	Mean blood pressure, mm Hg				Glomerular filtration rate, ml/min.				Renal blood flow, ml/min.				Renal vascular resistance*				Dog wt, kg	Dose
	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3		
Sub-group A																		mg
1	126	131	140	147	38	27	34	38	177	216	216	196	.71	.61	.65	.75	13.0	.2 (Total)
2	158	157	104	115	35	35	31	30	198	194	222	218	.80	.81	.47	.53	16.5	.2 "
3	132	132	126	142	37	33	41	35	202	200	234	198	.65	.66	.54	.72	11.0	.2 "
Mean	139	140	123	135	37	32	35	34	192	203	224	204	.72	.69	.55	.67	13.5	.2
Sub-group B																		
4	163	159	163	160	62	57	56	66	318	363	402	342	.51	.44	.41	.47	18.0	.5 (Total)
5	135	136	139	152	64	65	64	66	437	452	354	289	.31	.30	.39	.53	18.8	.5 "
6	138	137	135	128	50	55	50	44	320	317	260	185	.43	.43	.52	.69	18.0	.5 "
Mean	145	144	146	147	59	59	57	59	358	377	339	272	.42	.39	.44	.56	18.3	.5
Sub-group C																		mg/kg
7	139	121	113	111	45	53	40	37	251	269	195	170	.55	.45	.58	.65	15.0	.5
8	123	88	90	97	49	47	40	44	328	354	316	250	.38	.25	.28	.39	18.5	.5
9	63	43	40	64	30	30	30	31	134	132	121	106	.47	.33	.33	.60	12.0	.5
10	138	123	95	107	50	50	47	39	266	292	313	202	.52	.42	.30	.53	14.0	.5
11	103	60	56	89	49	44	44	34	192	180	219	120	.54	.33	.26	.74	20.0	.5
12	116	102	66	85	47	47	33	25	283	276	192	157	.41	.37	.34	.54	12.5	1.0
13	162	132	137	144	37	35	33	33	204	169	135	132	.79	.78	1.01	1.09	12.0	1.0
14	159	136	138	129	83	87	74	55	495	562	459	317	.32	.24	.30	.41	22.0	1.0
15†	123	89	47	45	44	38	9	15	284	306	42	208	.43	.29	1.12	.22	11.0	1.0
16	119	106	105	101	27	26	25	26	185	146	112	116	.64	.73	.94	.87	13.0	2.0
17	141	102	113	132	48	44	42	41	313	281	233	200	.45	.36	.48	.66	15.0	2.0
Mean	126	100	91	100	46	46	38	35	267	270	212	180	.50	.41	.54	.61	15.0	1.0
% of control		79	72	79		100	83	76		101	79	67		82	108	122		
P value‡ (<)		.001	.001	.001		NS	.05	.01		NS	.05	.001		.01	NS	.05		

C = Avg of 3-10 min. periods; control. D_1 = Immediately after drug administration (avg of 2-10 min. periods). D_2 = One hr after drug. D_3 = 3 hr after drug.

* Renal vascular resistance $= \frac{\text{Mean blood pressure}}{\text{Renal blood flow}}$.

† Dog died 8 hr later.

‡ $t = \bar{x}\sqrt{\frac{n(n-1)}{Sx_2}}$ (Sub-group C). Statistical analysis by R. A. Seibert, Ph.D.

TABLE IIB. Effect of Mecamylamine on Water and Electrolyte Excretion.

Dog No.	Urine vol, cc/min.				Sodium excretion, meq/min.				Potassium excretion, meq/min.				Hematocrit			
	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3
Sub-group A																
1	.6	1.9	1.1	.9	45	75	42	62	15	18	30	38	39	42	45	43
2	.5	.3	.7	.7	18	13	14	12	22	18	16	19	38	38	45	49
3	1.0	1.0	2.1	2.0	103	85	223	81	26	26	38	33	39	40	41	44
Mean	.7	1.1	1.3	1.2	55	58	93	52	21	21	28	30	39	40	44	45
Sub-group B																
4	1.0	1.6	1.8	5.5	45	68	62	60	24	32	42	62	32	35	35	34
5	1.8	2.5	2.2	1.7	285	382	302	182	43	64	64	70	41	40	41	43
6	3.7	2.7	1.6	1.6	269	272	170	175	30	29	23	27	35	35	35	33
Mean	2.2	2.3	1.9	2.9	200	241	178	139	32	42	43	53	36	37	37	37
Sub-group C																
7	3.0	1.8	2.4	1.5	257	135	84	47	43	49	44	40	37	36	36	39
8	1.2	1.2	2.1	2.2	190	152	183	166	42	38	56	51	39	39	37	38
9	.5	.6	.6	.9	25	37	39	44	19	22	24	31	41	38	34	36
10	1.5	1.8	1.4	1.2	178	215	137	75	28	41	38	36	39	39	38	35
11	7.1	5.9	6.7	2.2	722	639	678	188	97	111	110	61	41	39	36	35
12	.5	.5	.4	.6	18	37	15	24	27	41	16	24	40	41	40	40
13	1.0	1.1	1.3	2.1	47	61	62	80	22	23	21	19	51	48	45	43
14	3.6	7.3	5.7	4.1	291	403	173	103	74	73	57	58	35	35	36	40
15	1.0	.7	.3	.4	87	58	6	9	40	25	1	5	43	47	45	47
16	2.1	2.3	1.2	1.6	123	186	93	136	29	39	30	45	35	35	32	32
17	1.1	.7	1.4	1.5	50	92	87	122	41	42	37	38	39	38	37	37
Mean	2.1	2.2	2.1	1.7	181	183	142	90	42	46	39	37	40	40	38	38
% of control		105	100	81		101	78	50		110	93	88		100	95	95
P value* (<)		NS	NS	NS		NS	.10	.10		.20	NS	NS		NS	.05	.20

C = Control (avg of 3-10 min. periods). D_1 = First 20 min. after drug administration (avg of 2-10 min. periods). D_2 = One hr after drug administration (avg of 2-10 min. periods). D_3 = 3 hr after drug administration (avg of 2-10 min. periods).

$$* \; t = \bar{x} \sqrt{\frac{n(n-1)}{Sx^2}} \text{ (Sub-group C).}$$

tion (decreased renal vascular resistance), was adequate ($p<0.01$) to compensate for the reduction in blood pressure. However, with prolonged hypotension, there was significant reduction in glomerular filtration rate and in renal blood flow ($p<0.05$). This was probably enhanced by the anesthesia and the fact that the ganglionic blockade persisted throughout the period of observation in contrast to hexamethonium, which is active for a much shorter period of time(2,3). The reduction in glomerular filtration rate was not accompanied by alterations in water and electrolyte excretion.

Clinical observations on acute responses to single doses (Group I): The average single dose which would reduce the blood pressure to approximately normotensive levels in the upright position when administered orally was 19 mg but the range was 5 to 40 mg. The duration of action varied from 12 to 48 hours with an average of 18 hours. There was a latent period of 20 to 70 minutes after parenteral administration of mecamylamine and 30 minutes to 2 hours after oral administration. Table III shows that the final response to equal oral and parenteral dosages was about the same. After an effective dose was given, increasing the dose had very little additional effect on the supine blood pressure but markedly enhanced and prolonged the upright pressure response.

Out-patient treatment of hypertension (Group II): For purposes of analysis, the 50 patients have been divided into 2 sub-groups on the basis of their upright control diastolic blood pressure. Sub-group I consisted of 9 patients with less severe hypertension, *i.e.*, their control upright diastolic blood pressure was greater than 100 but less than 120 mm Hg.

TABLE III. Summary of Data for Single Dosage Response to Mecamylamine in 12 Patients with Hypertension.

Patient		Control blood pressure Supine S	Control blood pressure Supine D	Control blood pressure Upright S	Control blood pressure Upright D	Blood pressure max response Supine S	Blood pressure max response Supine D	Blood pressure max response Upright S	Blood pressure max response Upright D	Onset blood pressure reduction (min.)	Duration blood pressure reduction (hr)	Dose (mg)*	Route	Side effects	Renal disease	Ocular fundi†	Age
D.D.	A	188	122	182	120	132	78	102	72	60	14	10	Oral	0	0	III	59
J.H.	A	220	140	200	130	200	130	140	100	90	16	15	"	0	+++	II	47
B.N.	A	230	110	230	110	150	100	70	50	90	24	5	"	+++‡	+++	II	61
F.H.	A	170	110	160	100	140	100	140	100	90	24	5	"	0	0	I	59
C.A.	A	200	120	190	110	180	110	110	80	30	14	20	"	0	++	II	56
P.M.	A	230	130	210	120	188	108	138	90	90	12	40	"	++‡	+++	III	58
J.R.	A	188	122	190	120	160	110	120	70	90	16	25	"	++‡	+++	III	66
T.T.	A	222	162	228	158	178	109	122	78	120	12	30	"	0	+++	III	44
	B	230	158	226	156	170	107	128	72	70	14	30	Intramusc.	0	—	—	—
T.R.	A	198	138	192	128	132	76	112	82	30	48	10	Oral	0	0	II	38
	B	188	140	190	120	140	72	118	78	60	48	10	Intramusc.	0	—	—	—
K.C.	A	232	144	218	148	190	118	138	108	90	12	40	Oral	0	+++	IV	34
	B	250	154	238	150	178	112	146	104	60	16	40	Intramusc.	0	—	—	—
J.M.	A	198	110	190	115	140	80	120	78	30	12	15	Oral	0	++	II	60
	B	186	112	188	112	146	88	116	76	20	15	15	Intramusc.	0	—	—	—
L.T.	A	236	138	225	134	190	106	128	88	120	18	10	Oral	N & V	+	II	42
	B	236	136	256	137	225	110	160	100	30	16	10	Intramusc.	N & V	—	—	—

* This dose (established by titration in 5 mg increments) would repeatedly reduce the blood pressure to normotensive levels while standing.
† Keith-Barker-Wagener classification.
‡ Side effects consisted primarily of marked weakness and dizziness (BN) when standing.
S = Systolic blood pressure. D = Diastolic blood pressure.
A = Observations after oral administration. B = Observations after parenteral administration using same dose as A.

TABLE IV. Comparison of Blood Pressure Response—Mecamylamine, Hexamethonium and Pentolinium.

	Mecamylamine		+ Rauwolfia + Hexamethonium		+ Rauwolfia + Pentolinium	
	No.	%	No.	%	No.	%
No. patients treated	50	100	75	100	75	100
Responsive	46	92	57	76	59	79
Normotensive	12	24	28	37	25	33
Unresponsive	4	8	18	24	16	21
Avg daily dose of responders	17 mg		2307 mg		341 mg	

Sub-group II consisted of 41 patients with more severe hypertension (*i.e.*, control upright diastolic pressures greater than 120 mm Hg). The criterion for response was a fall in the mean control blood pressure of 20 mm Hg or more, or a return to normotensive levels (<150/100 mm Hg) in the upright position. The overall response rate was 92% and 24% of the patients became normotensive on a total daily dose of 17 mg of mecamylamine. Of the 9 patients with control diastolic pressures less than 120 mm Hg but greater than 100 mm Hg (Sub-group I), 7 (78%) responded while 2 (22%) of these became normotensive on an average dose of 13 mg of mecamylamine daily. In sub-group II (control diastolic blood pressures greater than 120 mm Hg in the upright position), 39 (95%) of the 41 patients were responsive and 10 (24%) returned to normotensive levels on an average daily dose of 19 mg of mecamylamine. The fall in blood pressure was greatest in the upright position; however, there was also a significant reduction in the supine blood pressure.

It is difficult to evaluate the beneficial effects of blood pressure reduction due to the short period of treatment with mecamylamine. However, the most outstanding symptomatic improvements were relief of headaches and angina pectoris which were present in two-thirds of the patients prior to therapy. About one-half of the patients with congestive heart failure showed symptoms of improvement during this short course of treatment.

The most annoying side effects with mecamylamine were constipation, weakness and dizziness. Constipation was adequately controlled by the use of cathartics such as milk of magnesia, cascara, and mineral oil. Although constipation was not marked in most cases, it is a serious side effect and should be treated actively to prevent fecal impaction and ileus. After continued therapy for several weeks, this side effect either lessens or disappears in most patients. Weakness was present in 38% of the group. In most patients, this was not marked and was hard to evaluate. Dizziness was present in 40% at some time during the course of therapy. In some cases, this was due to excessive orthostatic hypotension although syncope was not observed. Dizziness was marked in 3 patients and in these it was probably due to over zealous treatment. Nausea was present at some time during the course of therapy in 10 (20%) of the patients but only 3 (6%) vomited. Several other less serious side effects such as blurred vision and dry mouth were observed in a number of the patients. Comparison of the side effects of therapy with mecamylamine and 2 other ganglionic blocking agents reveals that the major side effects of ganglionic blockade are qualitatively comparable with the 3 drugs.

Summary and conclusions. Mecamylamine produced the same cardiovascular and renal hemodynamic effects on dogs as have been previously observed with such autonomic blocking agents as hexamethonium and pentolinium. When given to patients with hypertension, it produced a consistent reduction in blood pressure which was most marked in the upright position. The effect was about equivalent when given orally as when given parenterally, the average daily dose being approximately 19 mg. The onset of action ranged from ½ to 2 hours, and lasted 12 to 48 hours. The response was quite variable from patient to patient, but within the same patient, it was reproducible, thus minimizing one of the chief objections to ganglionic blocking agents for the treatment of hypertension.

1. Moyer, John H., and Handley, C. A., *J. Pharmacol. and Exp. Therap.*, 1955, v113, 383.

2. Beyer, Karl, personal communication to authors.

3. Stone, Clement, personal communication to authors.

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An Isolated Human Voluntary Muscle Preparation.* (22048)

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The use of isolated mammalian and amphibian nerve-muscle preparations for the study of drug and ion interactions at the motor end-plate is well known. The object of this communication is to describe a method of utilizing isolated human muscle obtained from surgical operations for similar study. Fasciculi from various muscle groups have been tried, but so far the human intercostal, obtained at thoracotomies, has been used most often because of its small discrete fasciculi, short fibers, availability, and accessibility.

Method. After the muscle specimen was removed, a suitable fasciculus was dissected out and placed in a vial containing Krebs' solution, which had been saturated with 95% O_2 and 5% CO_2. The vial was closed and initially packed in chipped ice during the time it was transported from the hospital to the laboratory. Subsequently it was ascertained that chilling of the muscle was unnecessary, and this practice has been discontinued. Each fasciculus was about 0.5-1.0 mm thick and 10-15 mm long. It was suspended in a bath of Krebs' solution between 2 platinum electrodes as previously described(1). Tracings were usually about 20-30 mm in height. Because the nerve supply to the muscle cannot ordinarily be isolated, an attempt was made to stimulate the intramuscular nerves without at the same time stimulating the muscle fibres themselves by using a square wave of very short duration. The square wave stimulator was triggered by one of a pair of rocking mercury switches; the other mercury switch was used to stimulate the muscle fibres directly as before(1), and the switches were rocked to provide alternate direct and indirect stimuli at 10-second intervals.

The electrodes used to stimulate the intramuscular nerves were placed about 5 mm apart on either side of the muscle rather than longitudinally since Rushton(2) has shown that transverse current preferentially affects intramuscular nerves. To the same end 10%, rather than 5%, carbon dioxide in oxygen was used, since evidence has been presented(3) that conditions which lower the pH *in vitro* reduce the chronaxie of nerve relative to that of muscle in the human subject. In our experience the use of 10% carbon dioxide did favor the stimulation of intramuscular nerves selectively. It has been determined that a square-wave pulse of 0.01-0.07 millisecond duration and 5-10 volts (as measured across the electrode by an oscilloscope) will stimulate only the nerve endings in this preparation, and the contractions elicited by the indirect stimuli are equal to or slightly less than those from direct stimuli. Furthermore, contractions from indirect stimuli are blocked completely by 1-1.5 μg/ml of decamethonium bromide or 2.5-3.0 μg/ml of d-tubocurarine chloride.

There was some evidence that the action of C10 on this muscle was biphasic as previously reported for the rabbit lumbrical(1).

Summary. A preparation of human voluntary muscle suitable for studies on neuromuscular transmission *in vitro* is described and some of the actions of curare and of decamethonium on it are recorded.

* This work was in part supported by a grant from the National Institutes of Health of the U. S. Public Health Service. Crystalline d-tubocurarine chloride and decamethonium bromide were supplied by the Burroughs Wellcome and Co., Tuckahoe, N. Y.

1. Jenden, D. J., Kamijo, K., and Taylor, D. B., *J. Pharm. Exp. Therap.*, 1954, v111, 229.

2. Rushton, W. A. H., *J. Physiol.*, 1930, v70, 317.

3. Casselli, C., and Pinelli, P., *Arch. Fisiol.*, 1953, v531, 94.

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Effect of Amniotic Fluid on Cortisone Inhibition of Growth. (22049)

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Normal growth under certain conditions is inhibited by systemic cortisone. One outstanding example of this biologic property of cortisone is its ability to inhibit normal wound healing(1,2). The local applications of beef amniotic fluid have been observed to reverse completely this systemic action of cortisone on healing wounds(3). Accordingly it seemed advisable to determine the effect of beef amniotic fluid upon other growing cellular systems. For this purpose the newly hatched chick was selected as a model.

Methods. Newly hatched White Rock chicks, obtained from a local hatchery, were fed Purina chick starter and water *ad lib.*, while being kept in a chick brooder incubator in the laboratory. The chicks were divided into the following groups: (a) 37 chicks were allowed to grow normally for 18 days; (b) 36 chicks received 5 mg of Cortisone (Merck & Co.) subcutaneously daily for 18 days;† (c) 27 chicks received daily subcutaneous injections of beef amniotic fluid for 18 days. The dose was increased from 5 cc to 10 cc per day as the chicks grew; (d) 35 chicks received 5 mg of cortisone in addition to 5 to 10 cc of beef amniotic fluid per day for 18 days. All chicks were weighed at the beginning of the experiment and at 18 days. Amniotic fluid was not given on the 18th day.

Results. The average weight gain of each chick per group is illustrated in Table I. Fig. 1 shows a photograph of three 18-day-old chicks. The chick on the left received only cortisone. The chick in the middle received cortisone plus beef amniotic fluid. The chick on the right is a normal 18-day-old chick. This study indicates that the chicks receiving beef amniotic fluid in addition to cortisone are larger and better developed than their mates receiving cortisone alone; although not the equal of their normal mates. It is of interest to note that beef amniotic fluid administered alone did not promote or accentuate normal growth. In fact, the chicks of group (c) show less average weight gain than normal.

TABLE I.

Group	No. of chicks	Avg wt gain in 0-18 days (g)
A. Normal growth	37	136.2
B. Cortisone treated	36	28.9
C. Amniotic fluid treated	27	91.3
D. Cortisone & amniotic fluid	35	49.9

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† The Cortisone was kindly furnished by Merck & Co.

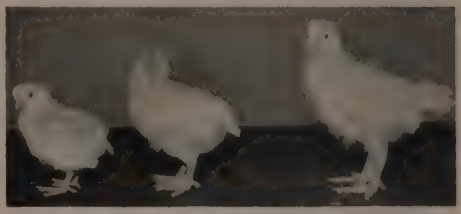

FIG. 1. Three 18-day-old chicks. Left chick given 5 mg cortisone daily, middle chick given cortisone plus amniotic fluid, right chick represents normal growth.

Beef amniotic fluid is known to contain growth-promoting and stimulating properties and is a satisfactory media for the tissue culture of mammalian cells. *In vitro* this may be the result of a ready supply of preformed nucleo-proteins(4-6).

With the possibility in mind that liver nucleo-protein may have been modified, liver sections from 8 chicks of groups (a), (b), and (d) were analyzed for their ribonucleoprotein content. The standard technic of toluidine blue staining before and after ribonuclease digestion of comparable sections was used(7). No differences in the liver ribonucleoprotein content were found by this technic in the livers from the 3 groups studied. It would seem unlikely that the liver content of nucleoprotein was effected by the procedures employed in the 3 groups studied.

Summary. Cortisone systemically administered to day-old chicks is a powerful growth

inhibitor for the 18-day period of this study. A partial reversal of this growth inhibitory activity of cortisone may be accomplished by the simultaneous administration of beef amniotic fluid.

I wish to thank Ovia Walker, Angela Ramos, and Tom Dillon for their technical assistance.

1. Howes, E. L., *Surgery,* 1950, v28, 177.
2. Spain, D. M., and Malomut, M., *Science,* 1950, v112, 335.
3. Montgomery, P., O'B., and Green, C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 657.
4. Fischer, A., *Nature,* 1930, v144, 113.
5. ———, *Chem. Products,* 1940, v3, 79.
6. ———, *Acta Physical Scand.,* 1942, v4, 207.
7. Herman, E., *Histochemical and Cytological Techniques,* 22nd edition, Department of Anatomy, Harvard Medical School.

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Effect of Temperature Variations on Paper Electrophoretic Migration of Serum Lipoproteins.* (22050)

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In a study on filter paper electrophoresis of serum proteins it became quite obvious that temperature changes markedly affected the migration of the serum lipoproteins even though the migration of the main serum proteins was affected to only a negligible extent. In order to determine what variations extremes in laboratory temperature might cause, we have carried out a study on the cholesterol distribution along filter paper strips following electrophoresis at 5°C versus summer room temperature and 35°C. The summer room temperature, though not known exactly, was probably around 26°C to 30°C most of the time.

Methods. A Kelab paper electrophoresis apparatus was used with Whatman #3 filter paper strips (4.6 x 35 cm) and a barbital-sodium acetate buffer of pH 8.6 and ionic strength .05. Three strips of filter paper separated from one another by plastic strips were placed between the glass plates of each of the 2 compartments, thus making a total of 6 strips for each run. To each of the strips at a line 12 cm from the cathode end of the strip a total of 0.05 ml of serum was applied evenly across the strip except for a margin of 2 mm at each edge. The duration of the electrophoresis was 15 hours. A current of 1.2 milli-amps per strip was employed consistently and the potential was approximately 210V. At the end of the electrophoretic procedure all strips were removed and processed as follows: a) 2 strips were oven dried at 100°C for about 45 minutes then stained with bromphenol blue for protein after the method of Köiw *et al.*(1); b) one strip was air dried then stained with Sudan Black-B for lipids; and c) the other 3 strips were kept from drying by suspending them in a 1000 ml glass cylinder containing water at the bottom and covered with an inverted petri dish to reduce evaporation. As soon as the position of the various proteins along the dried strips was determined, which seldom exceeded 90 minutes from the time the strips were removed from the electrophoretic apparatus, the wet strips were cut as indicated below and the sections transferred to 25 ml glass stoppered Erlenmeyer flasks. Nine sections were employed: 1) the albumin area, which also contained the alpha$_1$-globulin; 2) the area between the albumin, alpha$_1$-globulin and the alpha$_2$-globulin; 3) the alpha$_2$-globulin; 4) a narrow strip between the alpha$_2$- and beta-globulins; 5) the beta-globulin; 6) a strip between the beta-globulin and 5 mm from the starting point; 7) the area from 5 mm of the starting point to 5 mm on the other side of the starting point

* Grateful acknowledgement is made to the Reynolds Metals Co. for research grant and to Lederle Laboratories for summer research fellowship to PCT.

TABLE I. Effect of Temperature Variations on the Electrophoresis of Serum Lipoproteins.

No. of subjects	Avg % of total cholesterol/section with stand. dev.							Temp., °C.	Total chol.,* mg %
	Albumin to alpha$_2$	Alpha$_2$	Alpha$_2$ to beta	Beta	Beta to +5 mm	+5 mm to −5 mm	Gamma		
9	19.2±4.2 (12.7-27.4)	10.2±7.4 (2.5-23.3)	4.6±4.6 (0-12.6)	40.6±5.6 (34.3-48.7)	21.6±7.9 (8.2-31.9)	3.1±1.7 (0-6.1)	.6±1.0 (0-3.0)	35†	(175-272)
	18.2±3.6 (13.3-25.1)	3.3±1.4 (.9-5.8)	.2±.6 (0-1.9)	34.6±15.8 (6.5-55.6)	34.2±9.9 (24.5-50.7)	8.1±4.6 (2.3-17.0)	1.1±1.1 (0-4.1)	5	
4	11.1±1.6 (8.8-12.7)	18.4±14.5 (7.9-39.8)	8.3±3.3 (5.3-12.1)	43.2±8.4 (33.5-53.7)	14.4±2.0 (12.1-16.9)	3.7±3.1 (0-7.6)	.9±1.3 (0-2.7)	35†	(331-346)
	12.8±2.4 (9.4-15.1)	4.7±2.9 (2.3-8.8)	1.8±2.2 (0-4.4)	54.5±4.6 (51.5-61.3)	17.3±4.8 (10.4-21.5)	7.1±1.5 (5.7-9.0)	1.7±1.6 (0-3.2)	5	
1	4.9	2.0	6.8	24.3	41.2	19.2	1.3	35	529
	6.4	1.2	3.0	31.4	36.0	21.0	1.0	5	
1	6.5	32.4	3.8	24.4	21.5	9.2	2.1	35	674
	5.8	6.8	6.1	44.0	22.8	12.4	2.0	5	
Approx. width of strip (mm)	45	20	5	22	17	10	10	(blank = 10 mm)	

* Values below 300 mg % are considered normal for total cholesterol; total concentration determined by method of Forbes(2). (Range.)

† 2 in each of these were run at summer room temp. instead of 35°C.

which usually included some of the gamma-globulin; 8) the gamma-globulin; and 9) a portion 10 mm wide towards the end of the strip to serve as a blank. Approximately 17 ml of a 1:1 mixture of ethyl alcohol and ethyl ether were added to each flask, then stoppered, shaken and set aside overnight. On the following day the flasks were immersed to a depth of about 1 inch for 4 hours in a water bath at 63-65°C. Occasionally the solutions boiled slightly at first but sufficient pressure was never developed to blow the stoppers out. Most of the ether had escaped by the end of the period, at which time the flasks were removed from the bath. The solution remaining in each flask was transferred to correspondingly labelled test tubes and the paper and flask rinsed twice with about 3 ml of the alcohol-ether mixture. The tubes were then put in a water bath not above 63°C and the contents evaporated to dryness with the aid of a current of air. Each tube was removed from the bath as soon as dry.

Cholesterol determinations were made on each set of 9 tubes as follows: 2 ml of chloroform and 0.8 ml of acetic anhydride were added to each tube which was then shaken and put in a water bath at 22°C for 10 minutes; 1 drop of concentrated sulfuric acid was added, the tubes shaken, replaced in the water bath and set aside in the dark for 20 minutes for development of the color. The light absorption was read in a Klett-Summerson photoelectric colorimeter using micro cuvettes and light filter #42. A cholesterol standard was run simultaneously. Separate blanks were run for the standard and unknown solutions.

Some experimental results are shown in Table I. Since there was little cholesterol in section 2) the results thereof are combined with section 1). The values in the Table represent the average of 3 strips for each serum. The agreement between strips was quite good and the average of the sum of the 3 strips was usually in close agreement with the total cholesterol. The results on the various subjects with cholesterol below 300 mg per cent have been combined as are also those with values between 300 and 400 mg.

Although the rate of migration of the main serum proteins, as indicated by the bromphenol blue stain, was not significantly affected by the temperature variations employed, the rate of migration of the beta-lipoproteins was quite definitely affected as shown by both the Sudan Black-B staining and the cholesterol distribution. The temperature effect was especially marked in normal sera with normal cholesterol concentration.

The rate of migration of the lipids associated with the albumin, alpha$_1$-globulin area was not apparently affected by the temperature variations. It will be seen from the results shown in Table I that a fair amount of the cholesterol normally associated with the beta-globulin area moved considerably faster at 35°C than at 5°C. The amount of cholesterol remaining around the starting point was usually less at the higher temperature than at 5°C. This was especially evident in serums from normal subjects with normal cholesterol concentrations. The Sudan Black-B staining material in nearly all cases agreed quite well with the cholesterol distribution.

Discussion. The cholesterol distribution which we obtained at 5°C is in reasonably good agreement with that obtained by Boyd (3) using a temperature of 1-12°C. The deterioration of resolution of the alpha$_2$- and beta-globulins which Boyd obtained at higher temperatures was evident in only a few of our cases when electrophoresis was conducted at 35°C, and even in these cases the difference was not marked. The fact that the temperature differences did not apparently affect the rate of migration of the cholesterol-containing lipids found in the albumin, alpha$_1$-globulin area is probably a reflection of their high protein content and great stability as contrasted with the beta-lipoproteins.

Summary. Paper electrophoretic studies showed that the rate of migration of beta-lipoproteins was considerably less at 5°C than at summer room temperature or at 35°C. No effect of temperature was evident in the case of the lipoproteins migrating with the albumin, alpha$_1$-globulin. The rate of movement of the main serum proteins was apparently the same at the different temperatures, but the resolution of the alpha$_2$- and beta-globulins was sometimes less distinct at the higher temperature. The results presented show the importance of using relatively constant temperatures when comparative paper electrophoretic studies are carried out, especially of beta-lipoproteins.

1. Köiw, E., Wallenius, G., and Gronwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 47.

2. Forbes, J. C., *J. Lab. and Clin. Med.*, 1931, v16, 520.

3. Boyd, G. S., *Biochem. J.*, 1954, v58, 680.

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Effect of Hg^{203}-Labeled Mercaptomerin on Renal Sulfhydryl Concentration in Normal Rabbits.* (22051)

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When labeled organic mercurials are injected intravenously into normal animals, the radioactivity is localized in the kidney(1,2). The purposes of the present study were to measure the concentration of mercury in the kidneys of normal rabbits following the administration of large doses of the tagged mercurial, and to determine the effect of this agent on the renal concentration of sulfhydryl compounds.

Material and methods. Mercaptomerin sodium‡ was labeled with Hg^{203} to contain 40 μc of Hg^{203} per mg of mercury; each ml of the stock solution contained 40 mg of mercury in 140 mg of mercaptomerin sodium. Fourteen young rabbits were used. Each of 12 animals was anesthetized with ether and a unilateral nephrectomy was performed. The desired

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† Established Investigator of the American Heart Assn.

Hg^{203} supplied by Oak Ridge National Lab., Oak Ridge, Tenn., on allocation from U. S. Atomic Energy Commission.

‡ Hg^{203}-labeled mercaptomerin sodium was synthesized by the Wyeth Institute of Applied Biochemistry.

TABLE I. Distribution of Hg^{203}-Labeled Mercaptomerin Sodium in Rabbit Kidneys and Its Effect on the Sulfhydryl Content.

I.V. dose of mercaptomerin (mg Hg/kg)	Conc. of mercury (mg/g dry wt tissue)		Serum Hg conc. (μg/ml)	Conc. of sulfhydryl (mg/g dry wt tissue)	
	Cortex	Medulla		Control kidney cortex	Test kidney cortex
17.9	1.20	.20	78.5	12.9	8.6
16.7	1.90	.70	46.0	7.8	5.4
19.6	1.90	.70	42.9	10.7	8.4
20.9	2.40	1.40	85.7	12.5	5.7
22.0	2.60	.80	—	10.8	6.4
32.5	2.60	.30	—	8.6	7.8
14.8	2.70	.50	—	9.2	7.5
26.9	2.70	.90	74.0	9.3	5.8
20.9	2.80	—	—	10.1	5.3
14.3	3.10	1.50	—	11.0	7.1
30.2	3.10	.90	96.5	8.4	6.4
16.8	3.40	.60	32.5	9.6	6.7
0	0	0	0	11.3	12.2
0	0	0	0	8.3	12.2
Mean values	2.53	.77		10.08	6.76

dose of tagged mercaptomerin (ranging from 14 to 30 mg of mercury per kilo of body weight) was then immediately injected into a marginal vein of the ear, and ether anesthesia was continued for another hour. Exactly one hr after injection of the mercurial, the animal was killed and the remaining kidney was removed. Two animals were used as controls and were treated in the same manner as the test animals, except that the injection of the mercurial was omitted. The cortex and medulla were separated by gross dissection. Samples from each, weighing approximately 1 g, were weighed and homogenized to a 5% suspension (weight to volume) in a phosphate buffer at pH 7.5 in the Elvejhem-Potter glass homogenizer. Three 1-ml aliquots of each of the 5% homogenates were analyzed for sulfhydryl content by the method of Anson (3). The SH concentrations found in the tissues have been expressed in terms of the 1-cysteine equivalent per g dry weight of tissue. Another 1-ml aliquot of each of the 5% homogenates was placed in a test tube of known weight and was assayed for radioactivity content. The test tubes were then dried at 110°C until constant weights were reached. The precipitates obtained after the addition of trichloracetic acid were decanted free of supernatant fluid, and were also assayed for radioactivity content.

Results. Table I. The concentration of mercury in the cortex varied between 1.2 and 3.4 mg/g of tissue (dry weight), with a mean value of 2.53 mg/g. These figures are considerably higher than the values found in the medulla, which ranged from 0.2 to 1.5 mg/g, with a mean of 0.77 mg/g. From 90 to 98% of the radioactivity found in the homogenates was recovered in the trichloracetic acid precipitate; the mean value was 94%.

The sulfhydryl concentrations in the renal cortices removed before injection of the mercurial ranged between 7.8 and 12.9 mg, expressed as 1-cysteine/g dry weight of tissue, with a mean value of 10.08 mg/g. In all 12 instances in which labeled mercaptomerin was administered intravenously, the sulfhydryl concentration in the test kidney (5.3-8.6 mg/g, with a mean value of 6.76 mg/g) was lower than that in the control kidney. In the 2 control animals not given mercaptomerin, however, the SH values increased during the period of anesthesia. The results of the SH determinations on the renal medulla were not reliable, due to technical difficulties, and have not been reported.

Comment. That Hg^{203} has a predilection for the renal cortex is evident from the fact that cortical concentrations of mercury were, on the average, 3.3 times greater than those in the medulla. This distribution would be expected if the mercurial had localized in the proximal tubules, as others have found in histochemical studies(4). Since most of the radioactivity was recovered in the trichlor-

acetic acid precipitate, it can be inferred that the mercury was bound to renal tissue proteins.

The results of the present study show that the intravenous administration of organically bound mercury is associated with a decrease in the renal cortical concentration of sulfhydryl radicals.

Summary. Hg^{203}-labeled mercaptomerin sodium, in a dosage of 14 to 30 mg of mercury/kg of body weight, was injected intravenously into normal rabbits immediately following a unilateral nephrectomy, and the remaining kidney was removed one hour later. The mean cortical and medullary concentrations of mercury, were, respectively, 2.53 and 0.77 mg/g of dry weight of tissue. The mean sulfhydryl concentration in the control cortices was 10.08 mg/g of dry tissue, expressed as 1-cysteine; the mean value obtained one hour after administration of the tagged organic mercurial agent was 6.76 mg/g. The SH concentration of the cortex decreased in all 12 test animals following the injection of labeled mercaptomerin, and increased in the 2 uninjected control animals.

1. Aikawa, J. K., Blumberg, A. J., and Catterson, D. A., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 204.

2. Pitts, R. F., and Borghgraef, R., *Fed. Proc.*, 1955, v14, 115.

3. Anson, M. L., *J. Gen. Physiol.*, 1939, v23, 247.

4. Farah, A., Cafruny, E. J., and DiStefano, H. S., *J. Histochem. and Cytochem.*, 1955, v3, 271.

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Effect of Thyroxine and Related Compounds on Heparin-Activated Fatty Acid Liberating Enzyme.* (22052)

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Plasma from a human given intravenous sodium heparin contains an enzyme capable of hydrolyzing the triglycerides of egg lipoproteins and human S_f 20-400 lipoproteins, the latter both *in vitro* and *in vivo,* releasing fatty acids and increasing the S_f 4-20 lipoprotein level of the plasma(1-3). The lipoprotein transformations involve primarily the lipid components since the protein components of the S_f 4-20 and S_f 20-400 lipoproteins of normal humans seem to be of quantitatively identical amino acid composition(4). No definite evidence exists that the heparin-activated enzyme normally mediates lipoprotein metabolism, though Jeffries(5) has demonstrated the presence of a very similar, if not identical, enzyme in the rat following olive oil administration, and Korn(6) has obtained from rat heart an enzyme catalyzing the hydrolysis of the neutral fat of chylomicra. Though very much less susceptible to hydrolysis by the heparin enzyme, human S_f 4-20 lipoproteins can be significantly lowered in concentration by the administration of desiccated thyroid(7,8). For these reasons, and particularly if the heparin-activated enzyme is physiologically important (requiring for its activity no elements not normally present in the body), it seemed of interest to examine the effect of thyroxine and related compounds on the triglyceride-splitting enzyme.

Materials and methods. Enzyme (pre-heparin serum or post-heparin plasma) and human or egg lipoprotein substrate were prepared as described previously(1). L-thyroxine (THRX, obtained from Smith, Kline, French Laboratories and from Sigma Chemical Co.), L-triiodothyronine (TRIT, obtained from Smith, Kline, French), D-diiodothyronine (DIIT, obtained from Wyeth Laboratories, Inc.), and related compounds were dissolved in a minimum volume of 0.1 N sodium hydroxide and added to serum or plasma, the pH being adjusted to 8.0 with 0.1 N hydrochloric acid and the ionic strength made identical in all cases with 0.1 N sodium chloride. The calci-

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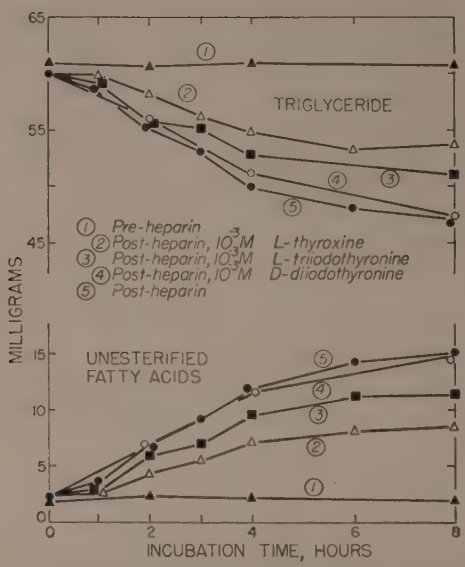

FIG. 1. Effect of thyroxine and related compounds on fatty acid liberation from egg lipoprotein triglycerides by post-heparin plasma.

um and magnesium experiments were carried out by adding the THRX-enzyme solution to substrate-cation solution and by adding THRX-enzyme-cation solution to substrate. In all experiments, after incubation of enzyme and inhibitor for 30 minutes at room temperature, the lipoprotein substrate was added and the contents incubated at 37.5°C. The pH remained constant in all experiments except for the 0.2-0.4 unit lowering brought about by the liberated fatty acid. At the end of the incubation, the contents were extracted with methanol-ethyl ether, the lipids separated chromatographically on silicic acid, and the fractions analyzed by infrared spectrophotometry(1).

Results. Effect of THRX and related compounds on fatty acid release. The data of Fig. 1 and of Table I indicate that THRX and TRIT inhibit the heparin-activated enzyme, the former compound being about 3 times more effective than the latter at equimolar concentrations. DIIT, L-tyrosine, and sodium iodide do not inhibit. Calcium or magnesium ion (3×10^{-3}M) or heparin (9×10^{-4} M, a concentration at least 400-fold that present in plasma after administration of 100 mg heparin) do not reverse the inhibition. In titrations carried out by the method of Alper(9), 3.9 ml 0.01 N sodium hydroxide were required to titrate the butyric acid liberated from tributyrin by post-heparin plasma, 3.7 ml that liberated by pre-heparin serum, 2.6 ml that liberated by post-heparin plasma 1×10^{-3} M in THRX, and 3.3 ml that liberated by post-heparin plasma 3×10^{-4} M in THRX. All the titrations were carried out in triplicate, with an uncertainty of 5-10%.

Effect of oral administration of THRX on fatty acid release and lipoprotein transformations. Before THRX administration, and after 10 mg intravenous heparin each day, the plasma of a normal individual at intervals of 2 days liberated 14.1, 13.4, and 13.9 mg fatty acid from 65 mg egg lipoprotein triglyceride prepared from different eggs. After daily oral administration of THRX (Smith, Kline, French tablets) for 8 days, 0.6 mg the first 2 and 1.0 mg thereafter, and following injection of 10 mg heparin at 2 times, the second 10 hours after the first, the plasma liberated 10.5 and 10.8 mg fatty acid, respectively from 66 mg lipoprotein triglyceride. During THRX consumption, the protein-bound iodine value rose from 5.6 to 14.9 μg/100 ml, corresponding at most to a THRX level of 2×10^{-6} M. At the conclusion of the experiment, THRX added to the plasma to a final concentration of 1×10^{-4} M was as effective in inhibiting fatty acid release from egg lipoprotein substrate as THRX added to a final concentration of 1×10^{-3} M had been before oral THRX consumption, 12.0 (10^{-3} THRX) and 12.5% (10^{-4} THRX) of the substrate triglyceride being hydrolyzed by THRX-containing, post-heparin plasma and 19.8% by the non-THRX-containing, pre-THRX administration post-heparin plasma.

The fasting standard S_f 0-20, 20-100, and 100-400 lipoprotein levels of the subject before THRX administration were 263, 54, and 21 mg%, respectively; after 10 mg intravenous heparin (Lederle) and withdrawal of the blood 10 minutes later, these values had become 288, 7, and 0 mg%. At the conclusion of THRX administration, these levels had become 129, 51, and 19 mg%, respectively; 10 mg intravenous heparin changed these levels to 144,

TABLE I. Liberation of Fatty Acid from Lipoprotein Triglycerides after Incubation with Pre-heparin Serum or Post-heparin Plasma and Various Compounds at 37.5°C for 4 Hours.

Incubation constituents	mg fatty acid increase*	$100 \times \frac{\text{mg fatty acid increase}}{\text{mg triglyceride in substrate}}$
(1) 2 ml S_f 20-400 lipoprotein (20-400 lp)	14.0	23.5
(2) 20-400 lp, 0.02 mg s. h.	.4	.6
(3) *Idem*	.5	.7
(1) " , 1×10^{-3} M L-thyroxine	7.6	12.7
(1) " , 3×10^{-4} M *Idem*	10.0	16.7
(1) " , 1×10^{-4} M "	12.0	20.1
(1) " , 1×10^{-3} M " , 9×10^{-4} M heparin	7.8	13.1
(2) " , 1×10^{-3} M "	.3	.5
(1) " , 1×10^{-3} M L-triiodothyronine	10.8	18.1
(1) " , 3×10^{-4} M *Idem*	12.3	20.6
(1) " , 1×10^{-3} M D-diiodothyronine	13.6	22.8
(1) " , 1×10^{-3} M L-tyrosine	13.7	22.9
(1) " , 1×10^{-3} M Sodium iodide	13.7	22.9
(2) " , 1×10^{-3} M L-triiodothyronine	.3	.5
(1) 2 ml egg lipoprotein (elp)	15.2	16.1
(1) elp, 1×10^{-3} M L-triiodothyronine	12.2	12.9
(1) " , 1×10^{-3} M L-thyroxine (THRX)	8.9	9.5
(2) " , 1×10^{-3} M THRX	.5	.6
(1) " , *Idem* , 3×10^{-3} M $CaCl_2$†	9.6	10.2
(1) " , " , 3×10^{-3} M $CaCl_2$‡	9.0	9.5
(1) " , " , 3×10^{-3} M $MgCl_2$†	9.3	9.8
(1) " , " , 3×10^{-3} M $MgCl_2$‡	9.3	9.8
(1) " , 3×10^{-3} M $CaCl_2$	16.0	16.9
(1) " , 3×10^{-3} M $MgCl_2$	15.5	16.4
(2) " , 3×10^{-3} M $CaCl_2$	.7	.8
(1) " , 1×10^{-3} M disodium versenate,§ pH 8.0	14.0	14.7
(1) " , 1×10^{-3} M sodium citrate, pH 8.0	13.9	14.6
(1) " , 1×10^{-2} M *Idem*	9.8	10.4

* The fatty acids produced came from the triglycerides, with no significant phospholipid or cholesterol ester hydrolysis to fatty acids. The values given represent averages of at least 4 determinations, with a reproducibility of 5-10%.

† Cation added to enzyme before enzyme addition to substrate.

‡ " " " substrate " " " " " .

§ Disodium versenate = disodium salt of ethylene diamine tetracetic acid dihydrate.

(1) = 3 ml post-heparin plasma; (2) = 3 ml pre-heparin serum, s. h. = sodium heparin and present in all incubations with (2); (3) = 3 ml pre-heparin plasma, obtained by drawing blood into a heparinized syringe.

21, and 6 mg%. All the experiments on fatty acid release and lipoprotein transformation ability were repeated with two volunteers given 3 grains of desiccated thyroid (Armour) daily for 3 days (until complaint of unpleasantness); at most, a 5-10% inhibition was observed though the S_f 0-20 lipoprotein level had been reduced 15%.

Discussion. The approximate 3-fold superiority of THRX to TRIT in inhibiting fatty acid release is in conflict with the usual relative effectiveness of these compounds in biological systems, the latter being at least as effective and quite often 2 to 10 times more effective in mediating reactions(10). In view of the antagonism between the iodinated amino acids and magnesium (THRX binding the cation more strongly than does TRIT (11)), and in view of the role of calcium and magnesium cations, albumin(12-14), or lipoproteins(15) as fatty acid acceptors and facilitators of triglyceride hydrolysis, the experiments with calcium, magnesium and THRX were performed. Failure after cation addition to reverse significantly the thyroxine-mediated inhibition argues against a calcium or magnesium ion-amino acid complex as the cause of the inhibition. In addition, the data in Table I indicate that the potential cation-complexing agents, citrate and versene (10^{-3} M) are much less effective than the iodinated amino acids (10^{-3} M) as inhibitors of fatty acid release. Since heparin addition does not reverse inhibition, the amino acids probably

do not function by heparin removal.

Many workers(16-21) have presented data that there appears to be an α-globulin component in human serum with a binding mechanism so specific for THRX that TRIT fits less well and DIIT is excluded. If the heparin-activated enzyme is of such nature that THRX is bound to it more strongly than TRIT, or if the concentration of the latter available for inhibition is reduced by combination with non-enzymic plasma proteins, then the latter amino acid could be less inhibitory than the former. Our kinetic studies are not of sufficient accuracy to indicate amino acid-enzyme or amino acid-substrate binding. Inhibition of both tributyrin and lipoprotein triglyceride hydrolysis may be a reflection of non-specific esteratic enzyme site binding. The relatively high concentrations of THRX needed for inhibition in our experiments and the failure to observe an increase in S_f 20-400 lipoprotein levels accompanying the decrease in S_f 4-20 levels in patients given desiccated thyroid(7) —the increase to be expected if the thyroid is a natural antagonist of the heparin enzyme and if the enzyme itself is physiological—make it premature at present to associate the observed thyroxine effects with physiological thyroid function. Cairns and Constantinides (22), using amounts of THRX far greater (0.2 mg/rat/day for 8 days) than those used by us, found that THRX inhibited the "lipemia-clearing activity" induced by heparin injection, and that thyroidectomy alone failed to accelerate the activity, concluding that the THRX effect was not physiological.

Summary. 1. L-thyroxine and L-triiodothyronine inhibit the heparin-catalyzed release of fatty acid from lipoprotein triglycerides, the former inhibiting significantly at 3×10^{-4} M and being about 3-fold more effective than the latter at equimolar concentrations. 2. The inhibition does not result from removal of calcium or magnesium ions or heparin.

We have profited from discussions with Drs. J. W. Gofman and T. R. E. Pilkington. We wish to thank Smith, Kline, French Laboratories (L-thyroxine and L-triiodothyronine), Lederle Laboratories (sodium heparin), and Wyeth Laboratories (D-diiodothyronine) for their generous gifts of pharmaceuticals.

1. Shore, B., Nichols, A. V., and Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

2. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S., *Circulation,* 1951, v4, 666.

3. Gofman, J. W., De Lalla, O. F., Glazier, F., Freeman, N. K., Lindgren, F. T., Nichols, A. V., Strisower, B., and Tamplin, A. R., *Plasma,* 1954, v2, 413.

4. Shore, B., and Shore, V., *ibid.,* 1954, v2, 621.

5. Jeffries, G. H., *Quart. J. Exp. Physiol.,* 1954, v39, 261.

6. Korn, E. D., *J. Biol. Chem.,* 1955, v215, 1.

7. Strisower, B., Gofman, J. W., Galioni, E. F., Almada, A. A., and Simon, A., *Metabolism,* 1954, v3, 218.

8. Strisower, B., Gofman, J. W., Galioni, E. F., Rubinger, J. H., O'Brien, G. W., and Simon, A., *J. Clin. Endocrin. and Metab.,* 1955, v15, 73.

9. Alper, C., *Standard Methods of Clinical Chemistry,* Academic Press Inc., 1953, v1, 71.

10. Barker, S. B., *Brookhaven Symposia in Biology,* 1955, v7, 74.

11. Lardy, H. A., *Recent Progress in Hormone Research,* Academic Press Inc., 1954, v10, 150.

12. Robinson, D. S., and French, J. E., *Quart. J. Exp. Physiol.,* 1953, v38, 233.

13. Gordon, R. S., Jr., Boyle, E., Brown, R. K., Cherkes, A., and Anfinsen, C. B., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 168.

14. McDaniel, R. A., and Grossman, M. I., *ibid.,* 1955, v89, 442.

15. Laurell, S., *Scand. J. Clin. and Lab. Invest.,* 1955, v7, 28.

16. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R., *Nature,* 1952, v169, 19.

17. Winzler, R. J., and Notrica, S. R., *Fed. Proc.,* 1952, v11, 312.

18. Larson, F. C., Deiss, W. P., and Albright, F. C., *Science,* 1952, v115, 626.

19. Deiss, W. P., Albright, E. C., and Larson, F. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 513.

20. Robbins, J., and Rall, J. E., *J. Clin. Endocrin. and Metab.,* 1954, v14, 772.

21. Robbins, J., Rall, J. E., and Petermann, M. L., *J. Clin. Invest.,* 1954, v33, 959.

22. Cairns, A., and Constantinides, P., *Canad. J. Biochem. and Physiol.,* 1955, v33, 530.

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Morphological Changes in Thymus of Rats Following Whole-Body Exposure to Massive Doses of Radiation.* (22053)

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During the course of studies on the biochemical effects of ionizing radiation we observed that more thymic tissue was present in young rats after exposure to over 10,000 r of X-radiation than in comparable animals which had received lesser dosages. Following this observation, a study was made of the histological changes in thymus glands of rats given massive doses of radiation and attempts were made to elucidate the nature of the response. The present communication contains the results of experiments which have clearly shown that massive, whole-body irradiation induces only minimal cellular destruction in the thymus, in contrast to the more pronounced cytological changes that follow exposure to smaller dosages. In addition, they provide evidence to indicate that exposure of the head region plays an important role in the pathogenesis of the paradoxical cellular response in the thymus.

Materials and methods. For the studies the following general plan was used. Randomly selected groups of 5 to 10 rats were exposed over the entire body to X-ray or gamma radiation in doses between 400 and 30,000 r. The animals of these and all subsequent groups were sacrificed 24 hours after the completion of the exposures. The thymus, spleen, and adrenal glands were weighed, fixed in 10% formalin or Bouin's solution and, together with a number of pituitary glands and brains, were studied in detail by histological means. In a study of the localization of the tissues that govern the thymocytic response, groups of rats were exposed locally over the mediastinal region to graded doses of radiation and other groups of animals were given radiation over the body with the head shielded. To investigate the temporal aspects of this biological response, groups of rats were first given a small dose of total-body radiation and at varied times thereafter were re-exposed to massive doses. Twenty-five normal rats, together with groups of anesthetized and nonanesthetized irradiated animals were sacrificed for control purposes. Young female rats of the Sprague-Dawley strain were used. All were approximately 3 months of age and had weights of 200 ± 20 g. They were maintained throughout the experimental period in air-conditioned quarters with free access to Rockland rat pellets and water. X-radiation was administered at a rate of 320 ± 20 r/min. by a Keleket Therapy Unit operated under the following conditions: 250 KV, 15 ma, and filtration of 0.25 mm of Cu and 1.0 mm Al. Gamma radiation was administered from a Co^{60} source at a rate of 1,000 ± 50 r/min. To obtain localized exposure of the thymic area, the animals were anesthetized (sodium pentobarbital, 35 mg/kg) and irradiated within a 0.25-in. thick lead cylinder which had a single 1.0 by 0.5 in. aperture over the mediastinal region. To restrict gamma radiation to the body, the head was shielded with 0.62 in. of lead and the rays were collimated through concrete. There was less than 25% scatter of radiation to the head, with the highest dosage. The radiation fields and dose rates were calculated in air with phantoms in position. Measurements were checked with a high range Co^{60} rate meter, a 2,500 r Victoreen thimble chamber, $FeSO_4$, and one- and 2-phase chemical dosimeters.

Results. Single-dose, massive, whole-body irradiation. The mean weights of the thymuses of groups of rats at 24 hours after total-body exposure to doses of X-radiation from

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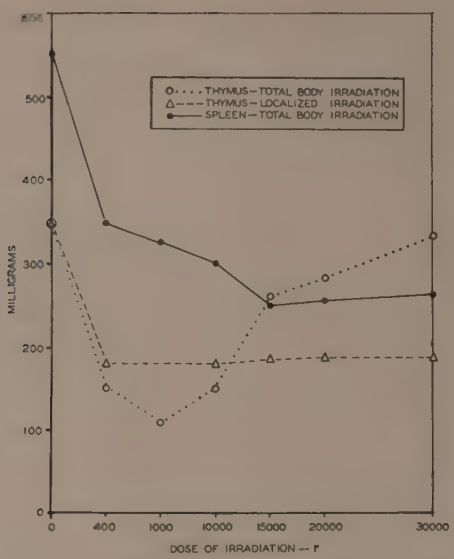

FIG. 1. Thymic and splenic weights in rats 24 hr after X-radiation of entire body and localized to mediastinal area. Each point is mean wt of organs from 5 to 10 animals.

400 to 30,000 r are shown graphically in Fig. 1. Following exposure to 400 r the thymus decreased within a 24-hour period to less than one-half its normal weight (mean weights, 361 to 149 mg). The weight loss was slightly greater after exposure to 1000 or 10,000 r. However, the administration of doses greater than 10,000 r was regularly followed by significantly less weight loss, with 30,000 r inducing only a 35 mg alteration in the mass of the thymus. A very close correlation existed between the weights of the thymuses of rats exposed to gamma radiation and those of animals administered the same quantity of X-radiation (Fig. 2). By contrast, the weight loss that was incurred in the spleens of these animals was regularly marked after all dosages, while the weights of the adrenal glands were not altered significantly from the normal. The weights of the thymus in anesthetized and nonanesthetized control groups were essentially identical.

In histological sections, the thymic tissues of rats exposed over the entire body to massive doses of X- or gamma radiation showed a conspicuous preservation of lymphocytes as compared with tissues of animals exposed to lesser dosages (Fig. 3). The preservation of cells was more evident in the cortex than in the medullary regions. In general, a very close correlation existed between the degree of cellular destruction, the width of the cortical layer, and the weight of the gland. The nuclei of many of the lymphocytes were pyknotic; nevertheless, the nuclear membranes remained intact and the chromatin material was particulate, being neither fused nor fragmented. Although karyorrhexis and phagocytosis were prominent features in the spleens of the same animals and in animals exposed to smaller dosages, they were inconspicuous or absent in the thymuses of rats exposed over the entire body to massive doses (Fig. 4). The adrenal and pituitary glands, as well as the central nervous system, with the exception of the granule cells of the cerebellum, showed no consistent morphological alteration attributable to radiation, the findings differing from those previously observed in monkeys(1).

Studies of the mechanism. When the mediastinal region of rats was exposed to localized X-radiation, the degree of cytological change

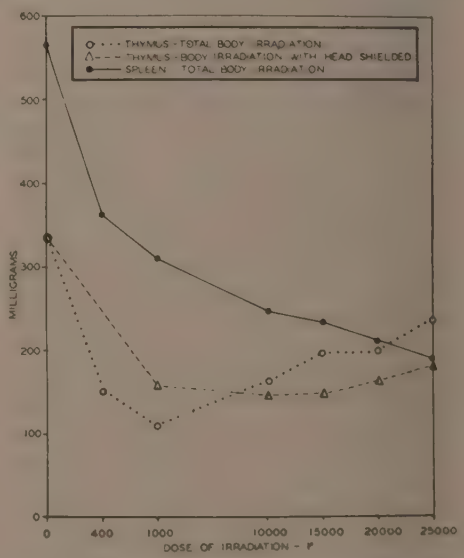

FIG. 2. Thymic and splenic weights in rats 24 hr after gamma radiation of entire body and localized to the body with the head shielded. Each point is mean wt of organs from 5 to 10 animals.

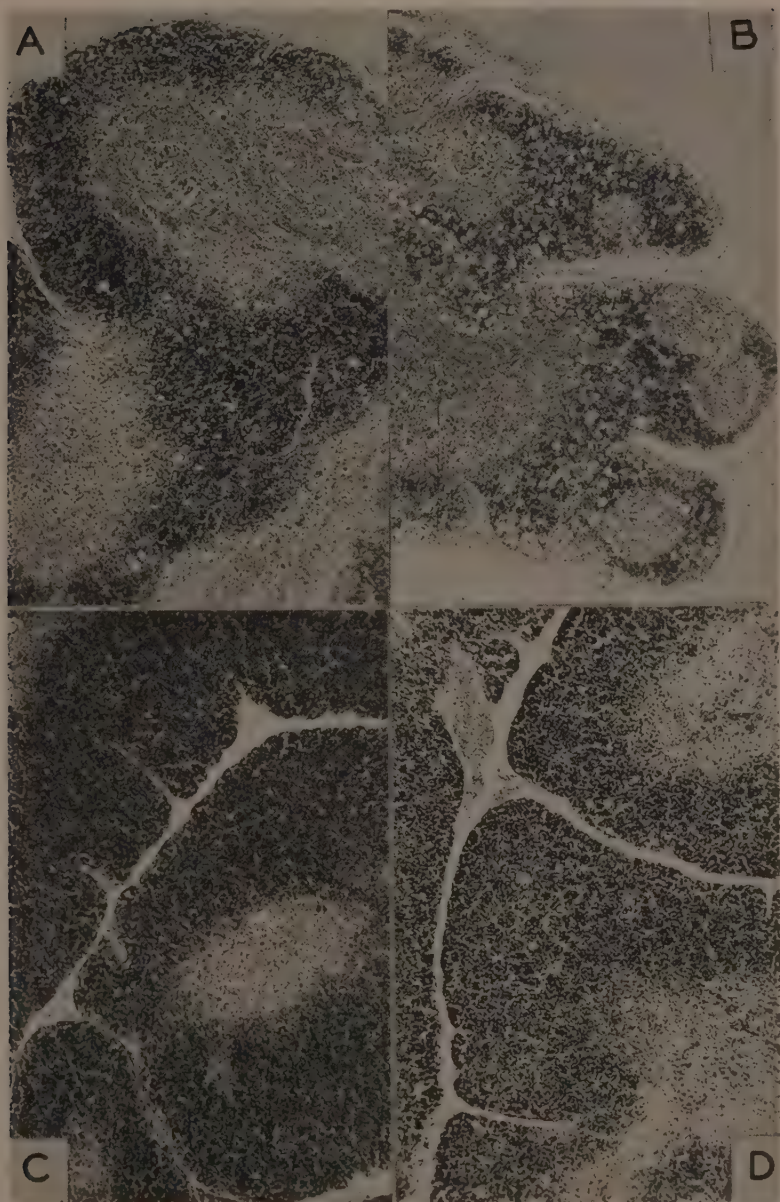

FIG. 3. Thymus of rats 24 hr after total-body exposure to X-radiation. Hematoxylin and eosin stain. × 50. A. (400 r exposure.) Cortex is narrowed, markedly depleted of lymphocytes, and has an avg thickness of .16 mm (normal = .52 mm). B. (10,000 r exposure.) Cortex is almost devoid of lymphocytes and has an avg thickness of .14 mm. C. (20,000 r exposure.) Cortex is of approximately normal thickness and is densely populated with lymphocytes. It measures on the avg .49 mm across. D. (30,000 r exposure.) Cortex is highly cellular and measures approximately .50 mm in width.

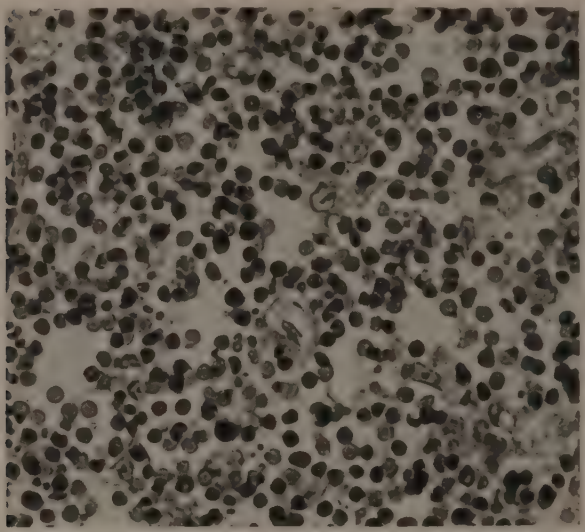

FIG. 4. (Higher magnification of Fig. 3D.) Lymphocytes in cortex are approximately normal in concentration although many are pyknotic. Karyorrhexis and phagocytosis are inconspicuous. Hematoxylin and eosin stain. × 800.

and weight loss in the thymus was essentially constant after all dosages above 400 r (Fig. 1). This finding provides evidence that the cytological response of the thymic lymphocytes to massive, whole-body irradiation is modified by the exposure of tissues outside the mediastinal area.

When the body alone was exposed to high intensity radiation with the head shielded, the degree of cytological destruction and weight loss in the thymus was significantly increased as compared with that induced by total-body exposure to the same dose of radiation (Fig. 2). This finding suggests that, during massive whole-body irradiation, exposure of the head plays an important role in the pathogenesis of the unusual cellular response in the thymus.

In groups of rats exposed to 400 r of whole-body radiation and then re-exposed within one hour to 19,600 r, the weight loss and cytological change in the thymus approximated that in animals exposed to a single, uninterrupted dose of 20,000 r. When the interval between exposures was lengthened to 6 hours, the weight loss in the thymus was markedly increased and equalled or exceeded that in animals exposed to a single dose of 400 r (Fig. 5).

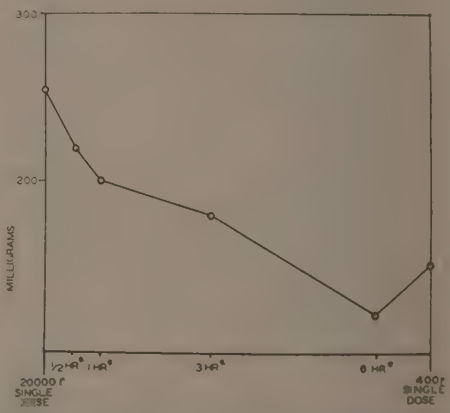

FIG. 5. Thymic weights in rats 24 hr after divided doses of 20,000 r of total-body X-radiation. Each point is the mean wt of organs from 5 to 10 animals. Asterisk indicates time lapse between administration of an initial dose of 400 r and a subsequent dose of 19,600 r.

Summary. The weight loss and degree of cytological alteration in the thymuses of rats was less after whole-body exposure to quantities of X- or γ radiation above 10,000 r than after exposure to smaller dosages. Administration of 10,000 to 30,000 r of localized radiation to the thymic region, or to the body with the head shielded, was regularly followed by

significantly greater cellular destruction and weight loss in the thymus than occurred after whole-body exposure to the same dose of radiation. Whole-body re-exposure to a massive dose of radiation within one hour after an initial total-body exposure to a small dose was regularly followed by less weight loss and cellular destruction in the thymus than occurred after total-body exposure to the small dose alone. The findings show that exposure of the head during massive whole-body irradiation markedly influences the cellular response in the thymus.

The authors wish to acknowledge the valuable assistance of Dr. Kenneth P. DuBois in these studies.

1. Haymaker, W., Vogel, F. S., Cammermeyer, J., Laqueur, G. L., and Nauta, W. J. H., *Am. J. Clin. Path.*, 1954, v24, 70.

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Effects of Oxidized Fatty Acids on Ascites Tumor Metabolism. (22054)

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The unsaturated fatty acids of cells and mitochondria have been shown to be oxidized by ultraviolet light(1,2) and by aerobic incubation(3). The oxidation products of these fatty acids quite probably alter cellular activities as indicated by the findings that the unsaturated fatty acid esters, methyl linolenate and methyl linoleate, inhibit the activity of certain oxidative enzymes in homogenates and mitochondria(2,3); depolymerize deoxyribonucleic acid (DNA)(4); and inhibit cell division(5,6). The relation of oxidized fatty acids to enzyme action is further examined in the present study of the action of oxidation products of linolenate and linoleate on the respiration and glycolysis of Ehrlich ascites tumor cells.

Methods and materials. Ehrlich ascites tumor growing in A-strain mice was taken from the abdominal cavity 7 days after injection. Blood-free tumor cell suspensions were prepared by centrifuging the ascitic fluid first for 10 minutes at 100 x g. The cells were then resuspended in Ringer solution and centrifuged at 2 x g for 15 minutes. The supernate which contains erythrocytes and platelets was discarded and the tumor cells resuspended in Ringer solution. This procedure was repeated 3 times. For testing the effects of oxidized fatty acids, aqueous extracts containing the oxidation products were made from pure fatty acid esters (Hormel) oxidized by irradiation with ultraviolet light. Samples of 0.5 g of the esters were irradiated for 90 minutes in dishes 3 cm in diameter placed at a distance of 30 cm from a Hanovia Utility Model mercury arc rated at an intensity of more than 250 microwatts/cm^2 at 50.8 cm distance for wavelengths of 3130 Å and shorter. 0.5 g of the irradiated fatty acid ester was dissolved in 10 ml of petroleum ether and extracted against an equal volume of distilled water with shaking for 20 minutes. After separation of the petroleum ether the aqueous phase was centrifuged under fresh petroleum ether to remove any suspended droplets of ether. The aqueous phase was then placed under vacuum for 20 minutes to remove traces of ether. Two volumes of the aqueous phase were diluted with one volume of 3 x isotonic Ringer solution and varying amounts of this mixture were added to Warburg flasks to give the desired amount of oxidized material as determined by the thiobarbituric acid (TBA) color reaction(7). The weight of oxidized ester cannot be calculated but is of the order of micrograms. The respiratory and glycolytic measurements were made by standard Warburg technics. Lactic acid formed aerobically was measured by the method of Barker and Summerson(8). Nitrogen was determined by digestion and nesslerization. Analysis of the unsaturated fatty acids of tumor cells was made on lipid of several grams of packed cells extracted with a 3:1 alcohol-ether mixture for 12 hours in a Soxhlet type extraction appara-

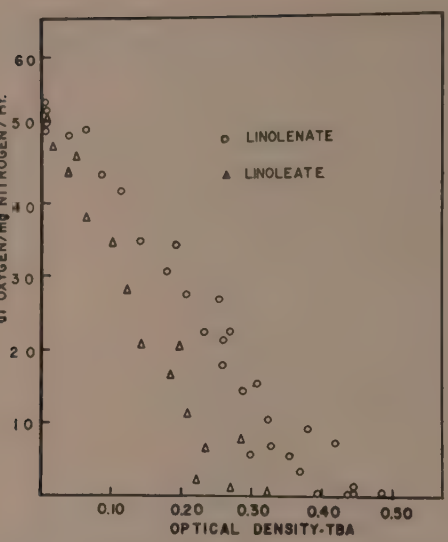

FIG. 1. Effects of aqueous extracts of oxidized methyl linolenate and methyl linoleate on respiratory activity of ascites tumor cells. Flasks contents: 1.0 ml of cell suspension containing about 5×10^7 cells; 2.0 ml of Krebs Ringer phosphate solution. Aqueous extracts of linolenate and linoleate were added with the Ringer solution. TBA values represent total amount of added oxidized esters. Measurements were taken for one hr.

tus. The percentage of each unsaturated fatty acid was measured after alkaline conjugation by the spectrophotometric micromethod of Herb and Riemenschneider(9). The iodine number was determined by the method of Benham and Klee(10).

Results. Respiratory inhibition. Washed tumor cell preparations exhibited a constant rate of oxygen consumption for an hour or longer followed by a gradual decrease in respiration over several hours. $Q_{O_2}(N)$ values during the first hour were between 50.0 and 54.5 as compared with a value of 58 reported by Kun *et al.*(11). The addition of small quantities of aqueous extracts of oxidized methyl linolenate and linoleate caused a decrease in respiratory activity which was a linear function of the concentration of extract (Fig. 1). The inhibition reached a maximum in the first intervals of the respiratory measurements. Inhibition by oxidized methyl linolenate was usually complete at concentrations giving TBA optical densities greater than 0.400. Extracts of oxidized linoleate inhibited oxygen consumption at lower TBA values than oxidized linolenate. However, since the TBA reaction is 30-80 fold more sensitive to oxidized linolenate as compared to oxidized linoleate(7), oxidized linoleate is actually a less potent inhibitor than oxidized linolenate.

Inhibition of glycolysis. Warburg and Hiepler(12) and Kun *et al.*(11) showed that ascites cells exhibited high rates of lactic acid formation in the presence of glucose under both aerobic and anaerobic conditions. The addition of aqueous extracts of oxidized linolenate to aerobic and anaerobic glycolyzing cells decreased the rate of lactic acid formation. The degree of inhibition was proportional to the amount of extract added in excess of a certain threshold concentration which was about the same for both aerobic and anaerobic glycolysis (Fig. 2). The inhibition appears to be slightly greater for aerobic glycolysis at higher concentrations of extract. It is to be noted that the respiration was inhibited completely at the threshold concentration for the inhibition of glycolysis (Fig. 1). The effect of oxidized methyl linoleate extracts followed the same general pattern observed for oxidized methyl linolenate (Fig. 3). The TBA values of oxidized linoleate extracts for any given degree of inhibition are lower than in the case of oxidized linolenate, but because of the difference in sensitivity of the TBA reaction to the 2 esters, higher concentrations of oxidized linoleate are required.

Fatty acid oxidation in the cell. The inhibition of respiration and glycolysis by oxidized unsaturated fatty acid extracts raises the problem of similar effects resulting from the oxidation of unsaturated fatty acids normally present in cells. Accordingly, attempts were made to oxidize the fatty acids within the Ehrlich ascites tumor cells under conditions which bring about fatty acid oxidation in other tissues. Also, analyses of the unsaturated fatty acid content of the cells were carried out. Tumor homogenates in Na-K phosphate buffer at pH 6.7 were shaken in air in a Dubnoff metabolic shaker, and after one hour the extent of lipid oxidation was measured by the TBA reaction. Different concentrations of homogenate were run over a

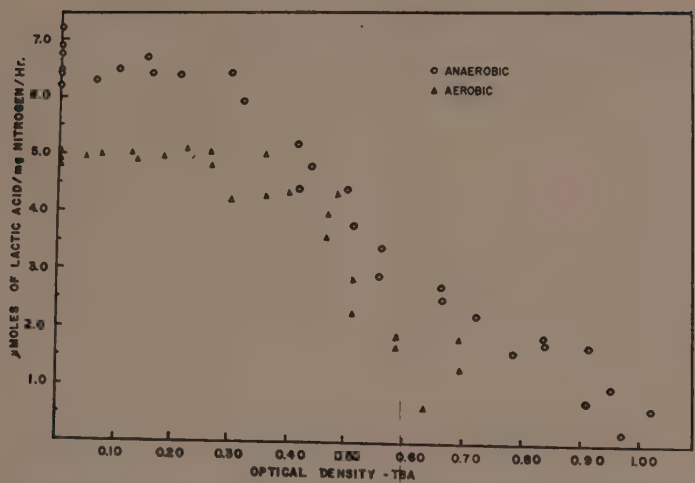

FIG. 2. Effects of aqueous extracts of oxidized methyl linolenate on anaerobic and aerobic glycolysis of ascites tumor cells. In anaerobic measurements each flask contained 1 ml of cell suspension containing approximately 2.5 × 10^7 cells, 2.0 ml of Krebs Ringer bicarbonate solution, and 0.2 ml of glucose solution (16.6 micromoles). Flasks were flushed with 95% N_2-5% CO_2 mixture, and lactic acid was measured by CO_2 evolution. In aerobic measurements Krebs Ringer phosphate solution was used; the flasks were flushed with 95% O_2-5% CO_2; and lactic acid was measured by chemical determination. TBA values represent total amount of added oxidized methyl linolenate. Measurements were taken for one hr.

range of 25 mg/ml to 250 mg/ml both with and without the addition of 0.3 mg/ml of ascorbic acid as a catalyst for lipid oxidation (13). Tumor homogenates were also irradiated with ultraviolet light and the oxidation products of fatty acids determined by the

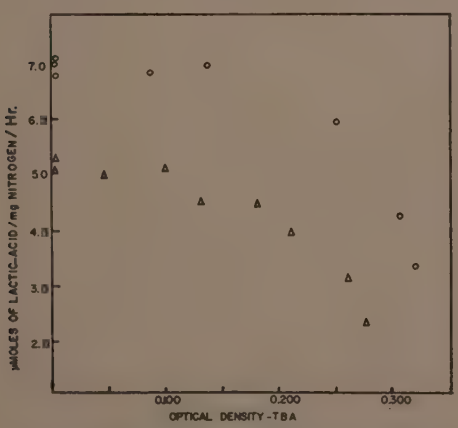

FIG. 3. Effects of aqueous extracts of methyl linoleate on anaerobic and aerobic glycolysis of ascites tumor cells. Experimental conditions as in Fig. 2. ○, anaerobic glycolysis; △, aerobic glycolysis.

TBA reaction. 5 ml of homogenate were exposed for intervals up to 2 hours slowly rotating in spherical quartz flasks 6.4 cm in diameter placed 26 cm from the mercury arc used to oxidize the fatty acid esters. The ultraviolet dose was greatly in excess of that required to oxidize lipids of mitochondria(2). In no case could significant fatty acid oxidation be detected. In contrast to tumor tissue, homogenates of mouse brain, liver, and kidney produced high values on incubation under identical conditions. Analyses of lipids of ascites cells are given in Table I. The figures are mean values of 3 replications on each of 2 lots of washed tumor cells, each consisting of the pooled tumor cells from several mice. The proportions of unsaturated fatty acids differ from rat liver(14) and also from rabbit bone marrow(15) analyzed by the same method. The concentration of linolenic acid is higher in the ascites cells than in rat liver and yet the TBA test is negative in the tumor and positive in liver(16). This indicates an increased antoxidant activity in the tumor.

Discussion. The inhibition of respiration of intact ascites cells by the oxidation products

TABLE I. Analysis of Unsaturated Fatty Acids of Ascites Tumor Cells.

Fat recovered/mg fat-free dry wt	.128 mg	
" " , wet wt	.019 "	
Iodine No.	72	70
Linoleic acid (%)	14.4	13.7
Linolenic " (%)	5.1	4.9
Arachidonic " (%)	4.0	3.9
Pentaenoic " (%)	3.2	3.3

of methyl linolenate and linoleate extends previous work on the inhibition of certain respiratory enzymes by these compounds(2,3). This study has shown that both aerobic and anaerobic glycolysis are also inhibited by the oxidation products of linolenate and linoleate. The observed effects on both respiration and glycolysis may involve a reaction between the oxidation products of fatty acids and sulfhydryl compounds(17). In view of the effects of oxidized fatty acids on the depolymerization of DNA(4) and on the hemolysis of erythrocytes(18), effects other than those involving sulfhydryl groups may be involved.

Analysis of the unsaturated fatty acids has shown that both linolenic and linoleic acids are present in the ascites cells. The oxidation products of both are inhibitory to cellular activities, and the oxidation products of linolenate (3 double bonds) are considerably more inhibitory than are the oxidation products of linoleate (2 double bonds). A similar difference has been noted in the studies of inhibition of bacterial growth(5) and the depolymerization of DNA(4). Within the cells a mechanism must be operative to hold in check the oxidation of these unsaturated fatty acids and the consequent effects of the oxidation products. Thus there is an absence of fatty acid oxidation products which would be inhibitory to cell division in the ascites tumor. Higher unsaturated fatty acids have also been shown to occur in ascites cells and in other tissues(14,15,19). The action of the oxidation products of these acids is not known.

Summary. 1. Extracts of irradiated methyl linolenate and methyl linoleate inhibited respiratory and glycolytic activities of Ehrlich ascites tumor cells. Respiration showed a greater sensitivity than glycolysis to the oxidation products of both esters. 2. The oxidation products of methyl linolenate inhibited both glycolysis and respiration at lower concentrations than those for methyl linoleate. 3. In contrast to the normal tissues of the mouse, the oxidation of tumor cell lipids by aerobic incubation and ultraviolet light could not be demonstrated by the TBA reaction. 4. Analyses of unsaturated fatty acids of ascites tumor cells are presented.

The author is indebted to Dr. K. M. Wilbur for his suggestions throughout the course of the work. The tumor was kindly supplied by Mrs. Elsie Siegel, Montefiore Hospital, N. Y. City.

1. Dubouloz, P., and Dumas, J., *Bull. soc. chim. biol.*, 1954, v36, 983.

2. Ottolenghi, A., Wilbur, K. M., and Bernheim, F., *Arch. Biochem. and Biophys.*, 1955, v56, 157.

3. Bernheim, F., Wilbur, K. M., and Kenaston, C. B., *ibid.*, 1952, v38, 177.

4. Fisher, W. D., and Wilbur, K. M., *J. Elisha Mitchell Soc.*, 1954, v70, 124.

5. Stillwell, E., *M. A. Thesis*, Duke University, 1952.

6. Wilbur, K. M., Kenaston, C. B., Wolfson, N., Ottolenghi, A., and Gaulden, M. E., *Anat. Rec.*, 1954, v120, 708.

7. Kenaston, C. B., Wilbur, K. M., Ottolenghi, A, and Bernheim, F., *J. Am. Oil Chemists Soc.*, 1955, v32, 33.

8. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, v138, 535.

9. Herb, S. F., and Riemenschneider, R. W., *Analyt. Chem.*, 1953, v25, 953.

10. Benham, G. H., and Klee, L., *J. Am. Oil Chemists Soc.*, 1950, v27, 127.

11. Kun, E., Talalay, P., and Williams-Ashman, H. G., *Cancer Res.*, 1951, v11, 855.

12. Warburg, O., and Hiepler, E., *Z. f. Naturforsch.*, 1952, v7b, 193.

13. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M., *J. Biol. Chem.*, 1948, v174, 257.

14. Johnson, R. M., Levin, E., and Albert, S., *Arch. Biochem. and Biophys.*, 1954, v51, 170.

15. Evans, J. D., Riemenschneider, R. W., and Herb, S. F., *Arch. Biochem. and Biophys.*, 1954, v53, 157.

16. Kohn, H. I., and Liversedge, M., *J. Pharm. Exp. Therap.*, 1944, v82, 292.

17. Dubouloz, P., and Laurent, J., *Bull. soc. chim. biol.*, 1953, v38, 819.

18. Rierson, H., 1955, unpublished results.

19. Holman, R. T., and Greenberg, S. I., *J. Am. Oil Chemists Soc.*, 1953, v30, 600.

Received September 13, 1955. P.S.E.B.M., 1955, v90.

Splanchnic Hemodynamics as Influenced by Hepatic Ischemia.* (22055)

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Certain hemodynamic observations made during a previous investigation in this laboratory(1) have shown that hepatic ischemia is followed by a vasodepressor response. The pertinent observations were a marked decrease in arterial pressure accompanied by elevation of portal venous pressure following 1 to 2 hours of complete hepatic ischemia. It was postulated in the preliminary paper that these manifestations could be attributed, in part at least, to hyperemia of the intestinal vasculature. Dilatation in the mesenteric circulation could cause, or contribute to the increase in portal pressure, and secondly, favor splanchnic pooling of blood which would contribute to the fall in arterial pressure.

The purpose of the present investigation was to study the portal circulation following complete hepatic ischemia of 2 hours' duration. Arterial, portal venous, and inferior vena cava pressures were measured with optical manometers concurrently with direct measurement of portal blood flow, so that hemodynamic calculations pertaining to vascular resistance changes could be made.

Methods. Experiments were conducted on 12 mongrel dogs anesthetized with 30 mg/kg of sodium pentobarbital intravenously. A midline incision was made followed by splenectomy. Residual splenic blood was drained into a heparin solution, filtered and reinfused into animals before beginning the experiment. A brass cannula of suitable size was inserted into the splenic vein until tip was near junction with portal vein. This cannula was connected by tygon tubing to a T-tube, one arm connected to a Gregg optical manometer (B), the other to inflow side of a Shipley-Wilson rotameter(2) (D in Fig. 1). The outflow side of rotameter was connected to a cannula introduced into the external jugular vein. A

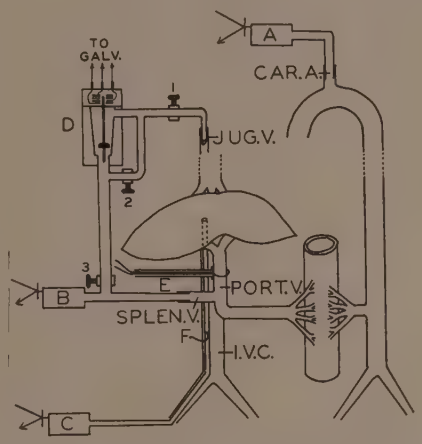

FIG. 1. Details circuit for measuring mesenteric blood flow. A, B, C: Optical manometers for measuring carotid arterial pressure, portal venous pressure, and inferior vena cava pressure respectively. D: Optically recording rotameter. E: Metal tube for ligature which occludes portal vein. Clamp 1 adjusts pressure in external circuit to agree with that registered in portal vein through cannula in splenic vein. Clamp 2 opens rotameter shunt circuit when necessary. Clamp 3 is opened when ligature at E is pulled taut, so that mesenteric blood flow is shunted into external circuit and the rotameter.

loose ligature was placed around portal vein as close to the liver as possible. This ligature was led out through brass tubing at E. Catheter (F) attached to manometer C was introduced via femoral vein into the inferior vena cava at level of diaphragm. This pressure was taken for hepatic venous pressure in some of the hemodynamic calculations. The other femoral vein was cannulated for infusion of fluids, additional anesthetic, and anticoagulant. Carotid blood pressure was measured with manometer A. The common hepatic artery was freed of investing tissue and nerve fibers, so that an occluding ligature could be passed around it. Care was taken not to interrupt the nerve fibers, and the ligatures were placed so as to lie between nerves and artery. The gastro-duodenal branch was ligated to minimize retrograde flow into the liver during

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TABLE I. Effect of Hepatic Ischemia on Splanchnic Hemodynamics.

	MABP, mm Hg			Flow, ml/min.†			R_M‡			PVP,§ cm/saline			HR§		
Exp. No.	A*	B*	%	A	B	%	A	B	%	A	B	%	A	B	%
1	116	95	−18	132	148	+12	.81	.58	−28	13.4	13.2	− 1	186	158	−15
2	148	92	−38	219	256	+17	.62	.32	−48	13.8	12.7	− 8	172	124	−28
3	158	138	−13	254	277	+ 9	.58	.46	−20	13.3	13.1	− 2	228	182	−20
4	138	142	+ 3	224	212	− 6	.57	.63	+11	13.7	14.3	+ 4	150	143	− 5
5	153	127	−17	159	161	+ 1	.88	.71	−20	17.8	17.0	− 4	200	177	−12
6	163	136	−17	188	190	+ 1	.85	.68	−20	12.2	12.2	0	209	197	− 6
7	162	150	− 7	207	157	−24	.74	.87	+18	15.7	16.1	+ 3	170	150	−12
8	160	155	− 3	208	191	− 8	.73	.77	+ 6	11.3	12.8	+13	212	190	−10
9	169	151	−11	137	110	−19	1.25	1.28	+ 3	14.8	14.1	− 5	182	160	−12
10	132	114	−14	243	183	−25	.51	.59	+16	12.0	10.5	−13	172	137	−20
11	161	159	− 1	259	262	+ 1	.58	.53	−10	13.2	15.3	+16	162	151	− 7
12	129	116	−10	183	173	− 6	.67	.64	− 5	9.1	9.2	+ 1	153	161	+ 5
Avg			−12			−4			−8			+.3			−15

* A, values before ischemia. B, values during final 30 min. of 2-hr ischemia.
† Flow does not include splenic contribution to portal vein.
‡ R_M = Mesenteric resistance, calculated from the ratio: $\frac{\text{MABP} - \text{PVP}}{\text{Flow}}$.
§ PVP = Portal venous pressure. HR = Heart rate.

ischemic period. Prior to beginning of experiment the animals were given a total dose of 40 mg heparin and 85 mg of a heparinoid (Manuronate); maintenance doses were continuously infused thereafter. The following method was used to measure *portal blood flow* preceding and following the period of hepatic ischemia. Portal venous pressure was noted immediately preceding each flow determination. The external circuit was then opened at clamp 3, and the ligature around the portal vein was pulled taut, occluding the vein against the brass tubing, E. The portal blood then flowed through the splenic cannula and the external circuit and rotameter into the jugular cannula. The caliber of tubing in external circuit was adjusted by clamp 1 so that pressure was maintained at exactly the same level as the portal pressure prior to manipulation, *i.e.*, while perfusing the liver. After stabilization, flow and pressures were recorded, then the circuit was closed and blood recirculated through the liver. The above procedure required less than a minute. Records were made at 2-3 minute intervals for an average time of 25 minutes. All flow and pressure measurements were made during the expiratory pause. Zero pressure reference was at the level of the inferior vena cava. No correction was made for intra abdominal pressure under the assumption that it would be approximately equal to the atmospheric pressure with the open abdomen. After initial flow and pressure measurements were made, the hepatic artery was closed and portal ligature at E pulled taut, so that portal blood was continuously diverted through the external shunt circuit for the 2 hour period of ischemia. Pressure in this circuit was kept at approximately the initial portal pressures with the aid of clamp 1. Flow measurements during the period of ischemia were made with continuous flow through the rotameter. Upon release of the occluding ligatures, flow measurements were resumed at first at 1-2 minute, later at 3-5 minute intervals, in the manner used prior to ischemia. Observations were usually continued for about one hour. During and following hepatic ischemia, glucose solution was continuously infused intravenously at the rate of 150 mg/kg/hr with a motor driven syringe.

Results. It is pertinent to present the hemodynamic changes which occurred during the 2 hour period of hepatic ischemia. The information is supplied in Table I, where mean arterial blood pressure, portal venous pressure, heart rate, portal blood flow, and mesenteric vascular resistance during the pre-ischemic period are compared with those obtained in the last half-hour of hepatic ischemia. Portal pressure was maintained at a constant value during the period, but all other functions

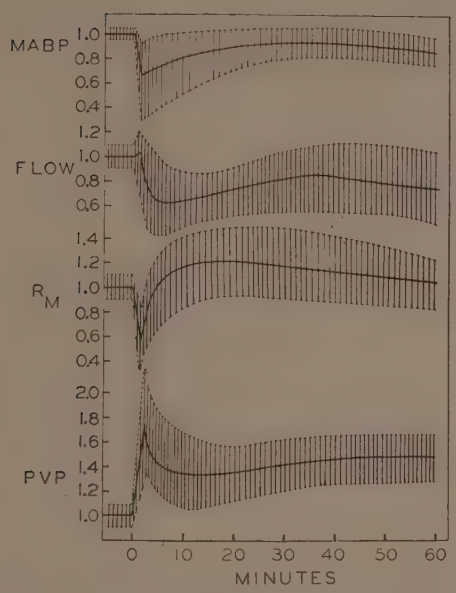

FIG. 2. Splanchnic hemodynamics following 2 hr of hepatic ischemia. Zero time coincides with time of release of ligatures occluding hepatic artery and portal vein. Solid line is avg trend of 8 experiments, expressed as ratios to function during the last 30 min. of the ischemic period. Dashed lines are drawn to indicate range of variation in this group of experiments and encompass *ca* 90% of all observations. MABP: mean arterial (carotid) blood pressure. Flow: portal (mesenteric) flow. PVP: portal venous pressure. R_M: mesenteric vascular resistance, calculated from the ratio $\frac{\text{MABP} - \text{PVP}}{\text{Flow}}$.

showed a tendency to decline by the following average values: arterial pressure, –12%; heart rate, –15%; blood flow –4%; and mesenteric resistance –8%. It was decided for greatest accuracy to compare post-ischemic manifestations with measurements made in the 30 minute period immediately preceding release of occluding ligatures. These values will serve as control values for the experimental changes induced by hepatic ischemia. A total of 8 experiments showed essentially comparable results. These experiments are summarized in Fig. 2 which shows the mean trends following ischemia as ratios to the 30 minute period immediately preceding release of the ischemia.

Mean Arterial Blood Pressure. This decreased rapidly upon release to 66% of the control in 1½ minutes. A gradual restoration of blood pressure followed, and at 35 minutes it had returned to 93% of control. Thereafter, a second downward trend ensued, so that pressure was 86% of control at end of the hour. In several animals which were followed beyond the hour, the downward trend was seen to continue, but in this series none was followed long enough to determine ultimate survival.

Portal blood flow. This showed a momentary tendency to increase during the first 1½ minutes, during which interval blood pressure was rapidly falling. However, flow soon decreased, reaching a value of 63% of control, 6 minutes after restoration of hepatic blood flow. It then gradually increased, attaining a maximal value of 86% of control at 35 minutes. Thereafter it decreased to 75% of control at 60 minutes.

Mesenteric resistance. This showed its greatest decrease 1½ minutes after release, when it was 56% of control, coinciding with the time of most marked decline of arterial pressure. This suggested that the fall in arterial pressure was caused by peripheral vasodilatation in which the mesenteric vessels participated. It is presumed that the drop in arterial pressure initiated compensatory vasomotor reflexes to cause reversal in blood pressure trend. Apparently, participating in compensatory vasomotor reflexes was the intestinal vasculature, for its resistance increased within 15 minutes to 21% above control, during which time arterial pressure showed its maximal rate of recovery. Subsequently, mesenteric resistance decreased somewhat, accompanying the secondary decline in arterial blood pressure.

Portal venous pressure. This increased abruptly on release of the portal vein ligature, attaining a maximal value of 75% above control during the brief period of minimal mesenteric resistance. It remained continuously elevated, however, at a somewhat lower value during remainder of experiment.

Heart rate. This showed no striking change in post-ischemic period, averaging 97% of control for the entire series. One dog, however, decreased to the range 73-87% of control for 14 minutes post-ischemia. In another,

heart rate decreased to 85% of control during the interval 4 to 11½ minutes post-ischemia. It is believed, however, that generally heart rate changes are non-contributory to changes in blood pressure. Four animals showed atypical results. One (Exp. 12) never attained a blood pressure higher than 65 mm Hg after ischemia, and died within 15 minutes after release of the ligatures. Portal pressure increased to 18.3 mm Hg, and mesenteric resistance averaged *ca* half the control value during the brief period of post-ischemia. Another (exp. 9) also failed rather soon; 30 minutes after restoration of hepatic circulation arterial pressure started a rapid decline, with concomitant decrease in mesenteric resistance indicating failure of compensation. Two other animals (Exp. 10 and 11) showed continuously increased resistance and reduced blood flow; no phase of reduced resistance could be detected following release of occluding ligatures. Blood pressure was well maintained in both. In these two, either a vasodepressor response was not produced by the liver ischemia or compensation was so rapid and complete as to be undetectable by our method of measurement.

Discussion. The transient phase of reduced mesenteric vascular resistance following restoration of the hepatic circulation attests to the probability that vasodilatation is a contributing component to the initial precipitous collapse of arterial pressure. The extent to which other vascular circuits participate in the response is not presently known. Two alternative mechanisms may be involved: activation of a vasodilator reflex, or release of vasodilator material from the anoxic liver. In consideration of possibilities of a vasodilator reflex, Alexander(3) has noted reflex mesenteric venous dilatation associated with pressure elevation in the inferior vena cava of dogs, but vena cava pressure in the present series showed no significant change following hepatic release. The possibility that increased portal venous pressure would initiate a vascular reflex was investigated(4), but no mesenteric dilatation was observed. Johnson(5) has noted a transient phase of reduced hepatic resistance immediately following transfusion after prolonged hypotension in rats, and has suggested that this may be a dilator reflex in response to elevation in arterial pressure when blood was re-infused. In our investigation, dilatation appeared to be localized in the mesenteric vessels, and concurrently hepatic resistance was increased.

The alternative mechanism, that a vasodepressor substance is released by the anoxic liver, is herewith favored, although based on circumstantial evidence. At present the nature of such a substance is speculative. VDM (vasoactive ferritin) had been proposed by Shorr *et al.*(6) as the vasodilator substance released by liver during anoxia of that organ, but conceivably other metabolites with vasodilator properties might be released in abnormal amounts following hepatic ischemia. Presenting difficulty to the hypothesis that a vasodilator substance is released by the anoxic liver is the fact that the organ itself apparently does not manifest hyperemia after 2 hours of ischemia. But vasomotor responses might be obscured by mechanical obstruction to flow incurred by the ischemia, *e.g.*, edema and swelling of hepatic lobular cells, with compression of the sinusoids.

The coincidence of the phase of reduced mesenteric resistance with the initial period of marked increase in portal pressure demonstrates that this hyperemia contributes to the observed elevation of portal venous pressure. But it is obvious that this is not the only factor—indeed, it may be a minor one—in sustained elevation of portal pressure, which remains elevated while mesenteric resistance is increased and intestinal inflow into the portal vein is reduced. The conclusion follows that the significant factor in elevation of portal pressure following hepatic ischemia is a sustained increase in resistance to portal inflow. If hepatic resistance is calculated from the ratio:

$$\frac{\text{portal pressure—inferior vena cava pressure}}{\text{portal flow}}$$

the ratio increases from 0.026 (.016–.039) (pre-ischemia) to 0.067 (.027–0.114) (post-ischemia) in 6 experiments of this group in which inferior vena cava pressures were measured. The latter pressure is taken as representative of hepatic venous pressure.

The compensatory reflexes which restore arterial pressure following initial collapse probably involve carotid and aortic pressoreceptors. In addition, it has been observed that elevation of portal pressure will itself cause mesenteric resistance to increase(4) by unknown mechanisms. The rapid institution of compensatory mechanisms makes it difficult to assess the true intensity and duration of the hepatic dilator action. The incomplete recovery of arterial pressure despite sustained increase in mesenteric resistance above the control value could be interpreted to mean that the influence of the hepatic depressor mechanism persists longer than the brief period of overt hyperemia indicates. The period of hyperemia could represent a phase of pooling and trapping of blood in capillaries and venules of the intestines (and possibly elsewhere), where it might remain segregated from active circulation, even though compensatory constriction of arterioles had supervened.

Hyperemic responses following anoxia and brief ischemia in other organs is a commonly observed phenomenon, but only a few illustrative examples can be cited here: coronary circulation(7,8), cerebral circulation(9), skin and muscle(10,11). It is generally opined that some metabolic substance having vasodilator properties is released in increased amounts as the result of anoxia to produce hyperemia, but the exact nature of the dilator substance is still a matter of speculation. Possibilities that have been invoked include the adenine nucleotides(12), histamine(10), and acetylcholine(8). When ischemia of the hind limb is prolonged (5-6 hours), renewal of circulation leads to circulatory collapse, the so-called "ischemic compression" shock (for refs. see (13)), presumably as the result of release of vasodepressor substances of a toxic nature by the ischemic limb.

Summary. The influence of complete hepatic ischemia of 2 hours' duration on arterial blood pressure, portal vein pressure, heart rate, and mesenteric vascular resistance was investigated in dogs. During ischemia, small gradual decreases occurred in arterial pressure, heart rate, portal blood flow and mesenteric resistance. On release of the occluding ligatures on the hepatic artery and portal vein, the following changes were observed: an immediate sharp decrease in arterial blood pressure; a brief period of increase in portal blood flow, followed by a decline; a sharp increase in portal venous pressure, and a marked decrease in mesenteric vascular resistance. These manifestations lasted approximately 5 minutes, and were then followed by a partial recovery of arterial pressure and portal blood flow, some reduction of portal venous pressure, and a significant increase in mesenteric resistance. Heart rate showed no significant change.

1. Selkurt, E. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 307.

2. Shipley, R. E., and Wilson, C., *ibid.*, 1951, v78, 724.

3. Alexander, R. S., *Fed. Proc.*, 1955, v14, 2.

4. Selkurt, E. E., *ibid.*, 1955, v14, 136.

5. Johnson, D. H., *J. Physiol.*, 1954, v126, 413.

6. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Circulation*, 1951, v3, 42.

7. Green, H. D., and Wegria, R., *Am. J. Physiol.*, 1942, v135, 271.

8. Eckenhoff, J. E., Hafkenschiel, J. H., and Landmesser, C. M., *ibid.*, 1947, v148, 582.

9. Schmidt, C. F., *Fed. Proc.*, 1944, v3, 131.

10. Lewis, T., *The Blood Vessels of the Human Skin and Their Responses*, Shaw & Sons, London, 1927.

11. Lewis, T., and Grant, R., *Heart*, 1925, v12, 73.

12. Green, H. N., and Stoner, H. B., *Biological Actions of the Adenine Nucleotides*, H. K. Lewis & Co., Ltd., London, 1950.

13. Wiggers, C. J., *Physiology of Shock*, The Commonwealth Fund, New York, 1950.

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Reversal of Fluoroacetate Inhibition by Vitamin B_{12} in a Strain of *Escherichia coli.* (22056)

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The addition of vit. B_{12} to aged cells of *Escherichia coli* strain 113-3, a mutant requiring vit. B_{12} or methionine for growth, stimulates the rate of oxidation of a number of substrates including acetate(1).

A study was conducted to investigate the cause of the stimulatory effect of vit. B_{12}. In the course of the work various metabolic inhibitors were used. Fluoroacetate was employed as an inhibitor of the tricarboxylic acid cycle, since it has been shown to be converted to fluorocitrate which acts as a competitive inhibitor of aconitase(2,3). The data reported here deal with the inhibitory effect of fluoroacetate on acetate and glucose oxidation in this mutant, and its reversal by vit. B_{12}.

Methods. The medium used was that of Davis and Mingioli(4) containing glucose and supplemented with 20 mg DL-methionine per liter. The organisms† were grown at 32°C for 18 hours without aeration in 18 liter pyrex bottles containing 16 liters of medium. The cells were harvested, washed twice, suspended in distilled water and aged in the refrigerator for a minimum period of 10 days. To adapt the cells to acetate, they were transferred in medium in which potassium acetate progressively replaced glucose. The acetate-grown mutant still required B_{12} or methionine for growth. The acetate-adapted cells were grown and treated in the same manner as the glucose-grown cells. The activity of the resting cell suspensions was measured at 37°C using conventional Warburg technics under air. All flasks contained potassium phosphate buffer, pH 7.0 (100 μM); vit. B_{12} (Cobione, Merck) was added at the same time as the substrate and inhibitor. The nitrogen content of the resting cell suspensions was determined by a micro-Kjeldahl method. Citric acid was determined by a modification of the method of Natelson, Lugovoy and Pincus(5).

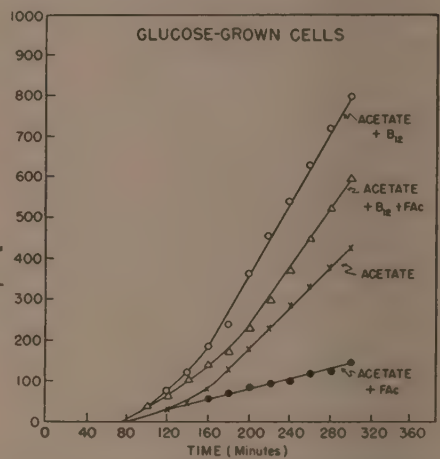

FIG. 1. Acetate oxidation in glucose-grown cells. *Flask contents:* 25 μM acetate, 3 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 1.09 mg. *Citric acid formed:* acetate ± B_{12}, 0; acetate + FAc, 0.1 μM; acetate + FAc + B_{12}, 0.3 μM.

Results. The effect of fluoroacetate‡ on acetate oxidation by glucose-grown cells is shown in Fig. 1. Acetate oxidation was inhibited and citric acid accumulated in the presence of fluoroacetate. No citrate was found in the absence of fluoroacetate. The addition of vit. B_{12} stimulated the oxidation of acetate as previously described(1), and decreased the inhibitory effect of fluoroacetate, although it did not affect the amount of citrate formed. The lag in acetate oxidation could be eliminated by the addition of malate. This may indicate that the main cause for the delay in oxidation of acetate is the need for the formation of a C_4-acid which is required for the oxidation of acetate via the tricar-

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† *E. coli* strain 113-3 was kindly supplied by Dr. Bernard Davis, New York University College of Medicine.

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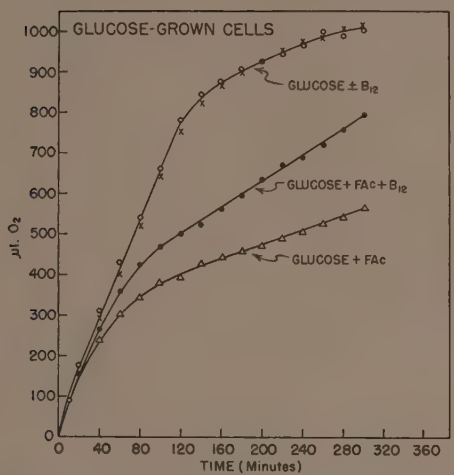

FIG. 2. Glucose oxidation in glucose-grown cells. *Flask contents:* 11 μM glucose, 5 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 1.77 mg. *Citric acid formed:* glucose $\pm$ B_{12}, 0; glucose + FAc, 0.3 μM; glucose + FAc + B_{12}, 0.4 μM.

boxylic acid cycle. Vit. B_{12} had no effect on this lag.

Since Ling and Chow(6) reported that vit. B_{12} was involved in glucose oxidation by erythrocytes, the influence of vit. B_{12} and fluoroacetate on glucose oxidation in this mutant was investigated. The results of this study are presented in Fig. 2. Vit. B_{12} had no effect on either the rate or extent of glucose oxidation. A marked inhibition of oxidation resulted from the addition of fluoroacetate, an effect which could be partially prevented by the addition of vit. B_{12} (Fig. 2).

The oxidation of acetate by acetate-grown cells and the effect of fluoroacetate and vit. B_{12} are shown in Fig. 3. No lag was observed in the oxidation of acetate, and vit. B_{12} had no effect on the rate of oxidation. The inhibitory effect of fluoroacetate was not as great as that found in the glucose-grown cells, and the addition of vit. B_{12} completely overcame it. Glucose oxidation was also inhibited by fluoroacetate, but it was only partially reversed by vit. B_{12} (Fig. 4).

The presence of fluoroacetate during the oxidation of glucose and acetate caused the accumulation of citric acid in both the acetate- and glucose-grown cells. Vit. B_{12} had no effect on the amount accumulated. In some cases the amount of citric acid found was slightly greater in the presence of vit. B_{12}. (See Fig. 1, 2, 3, 4).

Discussion. Fluoroacetate decreased the rate of oxidation of acetate and glucose by aged cells of *E. coli,* strain 113-3, which had been grown on glucose or acetate. The extent of inhibition was less in the acetate-adapted cells. Vit. B_{12} prevented this inhibition partially or entirely. The nature of the action of vit. B_{12} is not known. It would be possible to overcome partially the fluoroacetate inhibition by increasing the oxalacetate level or by introducing an alternate pathway of acetate oxidation. In this work malate, which would form oxalacetate on oxidation, did not replace vit. B_{12} in relieving the inhibition. If an alternate pathway is visualized, then acetate oxidation would by-pass the tricarboxylic acid cycle, although fluoroacetate would continue to form fluorocitrate which would accumulate and be determined as citrate. In these studies, although vit. B_{12} prevented some of the decrease in oxidation of acetate by fluoroacetate, it had no effect on the accumulation of citrate. It is unlikely, therefore, that vit. B_{12} affects directly or indirectly the enzyme systems of the tricarboxylic acid cycle.

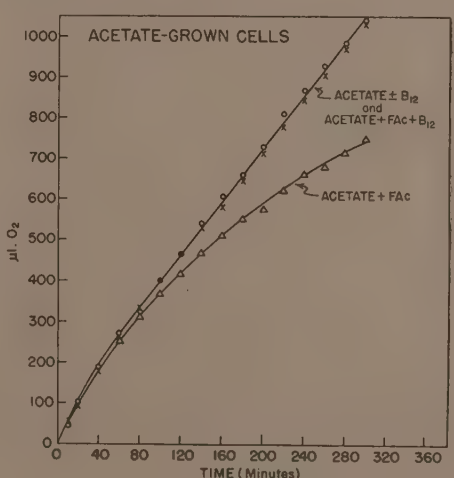

FIG. 3. Acetate oxidation by acetate-grown cells. *Flask contents:* 25 μM acetate, 3 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 0.7 mg. *Citric acid formed:* acetate $\pm$ B_{12}, 0; acetate + FAc, 0.6 μM; acetate + FAc + B_{12}, 0.5 μM.

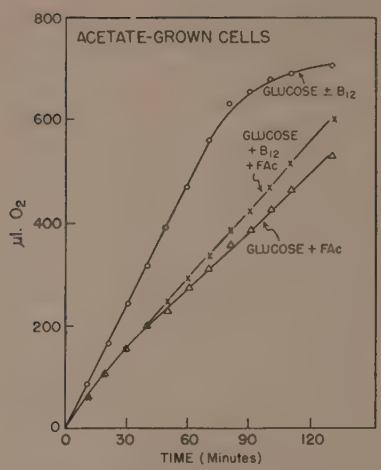

FIG. 4. Glucose oxidation by acetate-grown cells. *Flask contents:* 10 μM glucose, 5 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 0.7 mg. *Citric acid formed:* glucose $\pm$ B_{12}, 0; glucose + FAc, 0.6 μM; glucose + FAc + B_{12}, 0.5 μM.

The alternate pathway may be utilized to a greater extent in acetate-adapted cells, since there is less inhibition by fluoroacetate of acetate oxidation in these cells than in the glucose-grown cells. Vit. B_{12} may function by shifting acetate oxidation into the alternate pathway, either by stimulating the activity of an enzyme system or by stimulating the formation of the enzyme system, or one of its components. Since vit. B_{12} has no effect on acetate oxidation by mutant cells that are not aged, the aging process may cause a destruction or inactivation of some part of the alternate pathway. In acetate-adapted cells in which vit. B_{12} does not affect oxidation, less inactivation must occur.

Thus, as a possible explanation of the influence of vit. B_{12} on fluoroacetate inhibition, it is suggested that vit. B_{12} partially shunts the oxidation of acetate into an alternate terminal oxidative pathway.

Summary. 1. The rate of acetate oxidation in aged, glucose-grown cells of *Escherichia coli* strain 113-3 was stimulated by addition of vit. B_{12}. Stimulation of glucose oxidation was not observed. Upon addition of fluoroacetate with either glucose or acetate as substrate, oxidation was inhibited. This inhibition was partially prevented by the simultaneous supplementation with vit. B_{12}. 2. Acetate-adapted cells of this mutant failed to show a stimulatory effect of vit. B_{12} on oxidation of acetate. However, in the presence of fluoroacetate, oxidation of acetate and glucose was stimulated by vit. B_{12}.

1. Oginsky, E. L., Smith, P. A., Tonhazy, N. E., Umbreit, W. W., Lichstein, H. C., and Carson, S. F., *J. Bact.*, 1951, v61, 581.

2. Lotspeich, W. D., Peters, R. A., and Wilson, T. H., *Biochem. J.*, 1952, v51, 20.

3. Morrison, J. F., and Peters, R. A., *ibid.*, 1954, v56 (Proc.) xxxvi.

4. Davis, B. D., and Mingioli, E. S., *J. Bact.*, 1950, v60, 17.

5. Natelson, S., Lugovoy, J. K., and Pincus, J. B., *J. Biol. Chem.*, 1947, v170, 597.

6. Ling, C. T., and Chow, B. F., *Fed. Proc.*, 1954, v13, 253.

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Effect of Cholic Acid Administration on Taurine Concentration of Rat Organs.* (22057)

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The administration of cholic acid to growing rats causes a suppression of growth if the diet is low in sulfur amino acids(1). This effect can be reversed by feeding cystine, methionine or cysteine, but not taurine. In studying experimental atherosclerosis, Fillios and Mann(2) observed that rats fed cholesterol did not develop hypercholesterolemia if methionine was fed at the same time. White (1) points out that the cysteine deficiency observed after feeding cholic acid is the result of a diversion of cysteine into detoxification reactions. This is a plausible explanation; however, cholic acid is known to form mostly taurocholic acid in the rat(3,4), and endogenous taurine is used up in this reaction. It has been observed that the taurine concentration of rat organs remains constant even when animals are starved(5). Thus taurine must be formed from cystine, cysteine and to a lesser extent from methionine to replace the taurine consumed in the formation of taurocholic acid.

In this paper, data are presented which support the idea that taurine is formed from cysteine (or cystine) even when these amino acids are not supplied in the diet. In such a case, taurine is formed from endogenous cysteine, thus causing a cysteine deficiency.

Methods. Rats of the Sprague-Dawley strain were allowed to gain weight on a diet of Purina chow. When the animals weighed 200-220 g, they were divided in groups of 6 and each animal placed in an individual cage. Food consumption was determined daily. The diets used were prepared from a basic protein-free food mixture† of the following composition:

	%
Cornstarch	70
Vegetable oil	24
Salt mixture U.S.P.I.	4
Cod liver oil	2
Vit. supplement‡	

With this mixture we prepared diets containing 8 and 12% casein. Cholic acid diet was prepared by incorporating in the protein-free diet 8.16 g per kilo of cholic acid. A second diet was prepared containing in addition to the cholic acid 6 g per kilo of taurine. The animals were fed these diets for 10 days. At the end of this period, they were sacrificed and free taurine measured by a method recently reported(5). Liver, kidney, spleen, heart and muscle were studied.

Results. The absence of protein or the presence of either 8 or 12% casein in the diet has no effect upon the free taurine concentration of the organs studied (Table I). In Table II values are given for the taurine concentration of 5 organs from rats fed a protein-free diet and supplemented with cholic acid and cholic acid plus taurine. The values reported in these tables are within the normal range of values which we have reported for fed rats(5). None of the differences shown are significant.

Discussion. The early work of Foster, Hooper and Whipple(6) has already demonstrated that in the formation of taurocholic acid the limiting dietary factor was cholic acid and not taurine. The amount of taurine lost as taurocholic acid is not known precisely. The taurine excretion in the urine amounts to 50 to 80 micromoles per 24 hours(5) (6.2-10.0 mg). It is difficult to assess how much taurocholic acid is formed when animals are fed extra cholic acid in the diet. We believe that sufficient quantities of taurocholic acid are formed to create a deficiency in cysteine/cystine when these are present in limited amounts. The fact that no reduction in the

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† This was obtained from Nutritional Biochemicals.

‡ Vitamin Fortification mixture from Nutritional Biochemicals.

TABLE I. Effect of Dietary Protein on Taurine Concentration of Organs (Female Rats). (Micromoles taurine/g fresh tissue.)

Diet casein, %	Avg wt loss, g	Liver	Kidney	Spleen	Heart	Muscle
12	11	2.0 ± .4	8.3 ± .4	11.5 ± .5	25.0 ± .9	8.4 ± .4
8	14	1.6 ± .1	7.6 ± .4	11.6 ± .5	25.5 ± 1.5	9.2 ± .8
0	30	1.5 ± .2	9.4 ± .2	12.6 ± .4	25.6 ± .4	9.2 ± .7

Values are averages from 6 animals ± stand. error of the mean.

TABLE II. Effect of Cholic Acid Administration on Taurine Content of Rat Organs. (Micromoles taurine/g fresh tissue.)

Diet*	Avg wt loss, g	Liver	Kidney	Spleen	Heart	Muscle
			Males			
Basic (B)	24	1.0 ± .1	6.0 ± .6	12.2 ± .7	21.6 ± 1.4	8.5 ± .7
(B) + Ch	33	1.1 ± .5	6.2 ± .3	13.2 ± .3	27.1 ± .7	11.6 ± 1.3
(B) + Ch + T	30	1.0 ± .2	8.9 ± .5	13.6 ± .7	27.6 ± .9	12.6 ± .7
			Females			
Basic (B)	20	2.6 ± .4	9.1 ± .9	12.5 ± .4	28.1 ± 1.3	9.6 ± .2
(B) + Ch	38	1.8 ± .2	7.9 ± .5	12.0 ± .2	25.5 ± 1.5	7.7 ± .7
(B) + Ch + T	40	1.9 ± .2	10.2 ± .4	13.8 ± .8	30.3 ± .6	10.1 ± .8

* Ch = Cholic acid; T = Taurine.
Values are averages from 6 animals ± stand. error of the mean.

taurine concentration was observed in any of the group studied indicates that taurine was probably formed. If we consider the urinary excretion of taurine alone, the amount of cysteine, or methionine used in 10 days for taurine formation would be the equivalent of 62 to 100 mg. This is a considerable amount. Taurine fed to the animal has no effect in relieving symptoms of cystine/cysteine deficiency. Fed taurine is known to be rapidly excreted(7,8) and it is also possible that the rates at which taurine and cholic acid enter the liver are different. In order for the two to combine, they must be present at the same time at the site of the condensing enzyme. This is not very probable since both compounds differ markedly in properties. In Table II, we show that the addition of an excess of taurine over cholic acid (2:1 on a molar basis) fails to alleviate the effect of cholic acid upon growth, or to cause an increase in the taurine concentration of any of the organs investigated. Thus we must assume that taurine is formed at a fairly constant rate, and that its formation is independent of the amount of taurine present in the diet. Moreover, it can be concluded from this work that dietary taurine is not utilized for taurocholic acid formation to any great extent.

Portmann and Mann(9) have recently reported that taurine-S^{35} administered to rats gives rise to labeled bile salts. The greater portion of taurine, however, was excreted in the urine.

Summary. 1. Taurine concentration of 5 organs was measured in animals fed a protein free diet and diets containing 8 and 12% casein. No changes in taurine concentration were observed in any of the groups. 2. Administration of cholic acid alone or cholic acid plus taurine to rats maintained on a protein free diet had no effect on the taurine concentration of the 5 organs studied.

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1. White, A., *J. Biol. Chem.*, 1935, v112, 503.
2. Fillios, L. C., and Mann, G. V., *Metabolism*, 1954, v3, 16.
3. Bergstrom, S., and Gloor, V., *Acta Chem. Scand.*, 1954, v8, 1373.
4. Norman, A., *Acta Physiol. Scand.*, 1954, v32, 1.
5. Awapara, J., *Fed. Proc.*, 1955, v14, 175.
6. Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, v38, 367.

7. Schmidt, C. L. A., von Adelung, E., and Watson, T., *ibid.*, 1918, v33, 501.

8. Schmidt, C. L. A., von Adelung, E., Watson, T., and Allen, E. G., *ibid.*, 1920, v42, 55.

9. Portmann, O. W., and Mann, G. V., *ibid.*, 1955, v213, 733.

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Metabolism of Reserpine-C^{14}. II. Species Differences as Studied *in vitro*. (22058)

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The crystalline alkaloid, reserpine, which is currently finding use in the treatment of hypertension(1,2,3) and psychiatric disorders (4,5,6), was identified in extracts from the roots of *Rauwolfia serpentina* Benth by Müller, Schlittler and Bein(7). In a report on the pharmacology of reserpine, Plummer *et al.*(8) mention the wide range in doses necessary to elicit the characteristic tranquilizing response in the various laboratory animals. Since Sheppard, Lucas and Tsien(9) showed that the rat is capable of oxidizing the 4-methoxy carbon of the 3,4,5-trimethoxybenzoic acid (TMBA) moiety to carbon dioxide as well as hydrolyzing reserpine to yield TMBA, it was thought that the ability to degrade reserpine might be related to dose requirements. To test this hypothesis, liver slices of several animal species were tested for their ability to metabolize reserpine. Part of this work was presented at a meeting of the American Chemical Society(10).

Experimental. In vitro: The livers of mature rats, mice, pigeons and guinea pigs were obtained immediately after death from animals sacrificed for the purpose; while those from dogs and rabbits were obtained by biopsy. The livers were sliced freehand and incubated with 200 μg of reserpine-C^{14}. The preparation of fractions as illustrated in Fig. 1, and the methods of counting and synthesis of reserpine labeled with C^{14} in the 4-methoxy carbon of the 3, 4, 5-TMBA moiety are the same as those previously described(9). Subcellular particles of guinea pig liver were prepared according to the method of Schneider (11). Each fraction was incubated with 100 μg of reserpine-C^{14} for 3 hours at 37°C in Krebs bicarbonate buffer. The reaction was stopped with acid and the tissue extracted as previously described(9). *In vivo*: Four male guinea pigs weighing about 400 g were injected with reserpine-C^{14} in vehicle C(9). One animal received 1.0 mg/kg intravenously while the others received 0.5 mg/kg. $C^{14}O_2$ collections were made and counted as previously described(9).

Paper chromatography and radioautography. Aliquots of fraction C and C_B were mounted on Whatman No. 1 filter paper. 40 μg of TMBA were then added to each spot and the sheets chromatographed by descending method with a solvent composed of 5 normal ammonium hydroxide: butanol: isoproponal (3:2:1). Under these conditions TMBA gives an R_F of about 0.61 and fluoresces a bluish purple when exposed to ultraviolet light. To locate the radioactive spots radioautographs were made using Kodak No-Screen X-ray Safety Film.

Results. The results of the *in vitro* studies with liver slices are summarized in Table I. The rat possesses the greatest ability for the oxidation of the 4-methoxy carbon of the 3, 4, 5-trimethoxybenzoic acid moiety to carbon dioxide. Only the pigeon and rabbit seemed to be devoid of this activity. That most of this process depends on oxygen is obvious from the drop in $C^{14}O_2$ recoveries that occurred when incubation was carried out under nitrogen. The rat liver slices, however, show little ability for splitting off TMBA. Surprisingly, the guinea pig liver slices show a very marked ability in this respect, hydrolyzing about 80 per cent of the reserpine in three hours. The mouse and rabbit show fair hy-

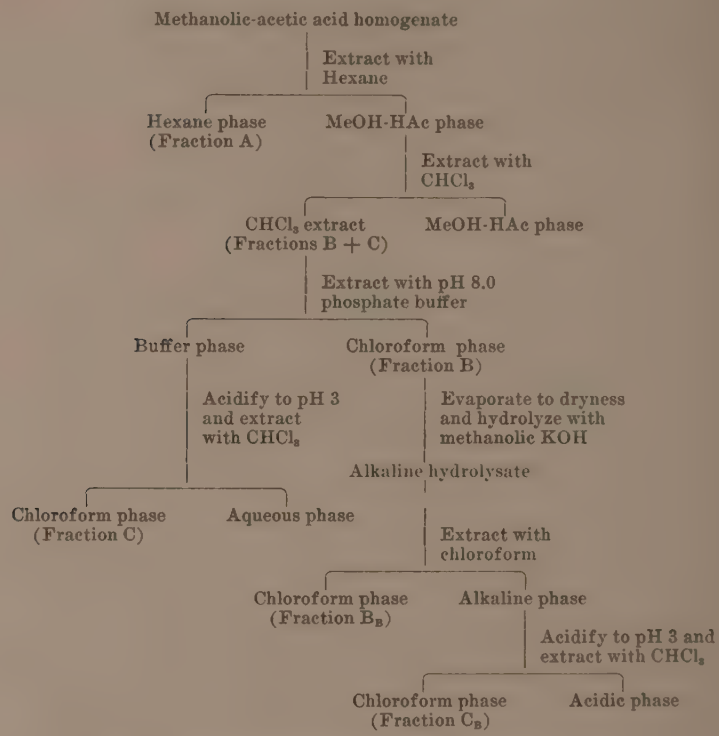

FIG. 1. Flow sheet for fractionation of liver homogenate.

drolytic activity while the dog and pigeon show practically none. The subcellular particles which possess most of the esteratic activity are the microsomes, as can be seen in Table II. The esteratic activity observed in the other fractions is less, and a portion is probably due to contamination with microsomes.

Radioautographs of paper chromatograms of Fractions C and C_B showed that all of the radioactivity coincided with the bluish purple fluorescence of TMBA.

The urinary excretion of C^{14} by the guinea pig is summarized in Table III. As can be seen, approximately 22% of the injected dose is excreted in the first 24 hours with some C^{14} still being excreted up to 96 hours. As confirmation of the *in vitro* studies indicating a slight ability to oxidize the 4-methoxy carbon of TMBA, some respiratory $C^{14}O_2$ could be detected.

Discussion. Among the animals used in these experiments, the dog is recognized as requiring the smallest and the rat the largest dose of reserpine to produce its typical tranquilizing response. It is apparent from the results presented that the dose requirement of reserpine is not directly related to the ability of liver slices to hydrolyze reserpine nor to demethylate the 4-methoxy group of TMBA or both. If it were, the pigeon would require the smallest and the guinea pig the largest dose of reserpine. The metabolic activity of other tissues must necessarily be considered when studying the response of the whole animal.

Because of the strong esteratic activity present in the guinea pig liver, it was supposed that the guinea pig would rapidly hydrolyze reserpine *in vivo* and excrete the TMBA via the urine. Therefore, it might be possible to demonstrate that pharmacological activity was lost when practically all of the TMBA was excreted, or, if some product of reserpine

TABLE I. *In Vitro* Metabolism of Reserpine-C^{14} by Liver Slices of Different Animals. 500 mg of liver slices incubated with reserpine-C^{14} in Krebs bicarbonate buffer at 37°C for 3 hr. All samples in duplicate.

Species	No. of animals	Atmosphere	% of added C^{14} recovered in fractions				
			CO_2	A*	B†	C‡	Total recovery
Dog	5	O_2	8.51	0	83.4	1.21	93.1
	1	N_2	0	0	96.2	.34	96.5
Pigeon	5	O_2	0	0	89.1	1.25	90.4
	1	N_2	0	0	86.0	1.28	87.3
Rabbit	5	O_2	0	0	80.1	15.7	95.8
	1	N_2	0	0	93.8	10.3	104.1
Mouse	5	O_2	5.6	.15	65.0	31.0	101.7
	1	N_2	—	—	—	—	—
Rat	5	O_2	19.7	.10	58.1	1.37	79.27
	1	N_2	1.3	.06	92.8	2.09	96.25
Guinea pig	5	O_2	2.67	1.51	3.19	80.6	87.97
	1	N_2	3.08	.62	2.25	91.8	97.75

* Fraction A—Contains fat-like substances.
† " B— " reserpine-like substances.
‡ " C— " trimethoxybenzoic acid-like substances.

were responsible for the pharmacological activity, such activity would continue beyond the time when all the TMBA were excreted. The results presented here not only do not support the latter supposition but also demonstrate that hydrolysis does not occur nearly as rapidly *in vivo* as *in vitro*. The reason for this great difference is probably that the reserpine is taken up by tissues which are not very active in metabolizing it(9).

As pointed out previously(9), the increase in the reserpine-containing fractions of rat liver slices incubated under nitrogen, indicates that demethylation probably occurs while TMBA is still esterified with methyl reserpate rather than after hydrolysis has occurred. This was confirmed again with the rat and also found to occur with dog liver slices.

The preponderance of esteratic activity found in the microsomes agrees with the findings of workers concerned with other esterases (12,13). Whether this esterase is a general or specific one remains to be determined.

The radioautograms demonstrate that the C^{14} content of fractions B and C reside in the TMBA moiety. It must still be demonstrated that the alkaloidal portion of the TMBA ester in fraction B is methyl reserpate. Thus far, paper chromatographic or electrophoretic studies fail to distinguish clearly between such alkaloids as reserpine, deserpidine or syringoyl methyl reserpate, indicating that small changes in the reserpine molecule would be extremely difficult to detect by this method.

TABLE II. Hydrolysis of Reserpine by Subcellular Particles of Guinea Pig Liver.

Fraction	% of added C^{14} present in fractions		
	B	C	Total
Homogenate	8.1	83.3	91.4
Nuclei	29.1	58.6	87.7
Mitochondria	35.3	52.5	87.8
Microsomes	9.0	91.9	100.9
Supernatant	10.6	75.7	86.3

TABLE III. Excretion of C^{14} in Urine and Feces of Guinea Pigs after Single Intravenous Injection.*

Rat No.	Interval, hr	Urine		Feces	
		B†	C‡	B†	C‡
1	1-24	.15	8.69	.68	.82
	24-96	.0	.35	.0	.0
2	1-24	.16	7.07	.58	.61
	24-96	.13	.43	.0	.0
3	1-24	.95	4.17	1.32	—
	24-96	.0	.83	.0	.0
4	1- 6	.73	21.9	1.62	1.66
	6-24	.13	1.13	.85	.15
	24-48	.07	.30	.26	.0

* Rats No. 1-3 received 0.5 mg/kg while No. 4 received 1.0 mg/kg.
† Fraction B—Contains reserpine and reserpine-like substances.
‡ Fraction C—Contains TMBA and TMBA-like substances.

Summary. 1. Liver slices of rat, mouse, dog, guinea pig, rabbit and pigeon were incubated with reserpine-C^{14}. 2. Demethylation of the 4-methoxy position of trimethoxybenzoic acid moiety of reserpine was observed to occur, with rat liver slices most active in this respect. 3. Hydrolysis of reserpine was observed to occur with liberation of free trimethoxybenzoic acid. Guinea pig liver slices were most active in this respect. 4. Esteratic activity resides chiefly in the microsomes of guinea pig liver. 5. Demethylation or hydrolysis or both by liver slices is not correlated with dose requirement for typical tranquilizing response. 6. Rate of excretion of C^{14} by the guinea pig was studied *in vivo* after intravenous injection of reserpine-C^{14}.

1. Wilkins, R. W., *Annals N. Y. Acad. Sci.*, 1954, v59, 36.

2. Hafkenschiel, J. H., and Sellers, A. M., *ibid.*, 1954, v59, 54.

3. Dustan, H. E., Taylor, R. D., Corcoran, A. C., and Page, I. H., *ibid.*, 1954, v59, 136.

4. Kline, N. S., *ibid.*, 1954, v59, 107.

5. Noce, R. H., Williams, D. B., and Rapaport, W., *J.A.M.A.*, 1954, v156, 821.

6. Weber, E., *Schweiz. med. Wschr.*, 1954, v84, 968.

7. Müller, J. M., Schlittler, E., and Bein, H. J., *Experientia*, 1952, v8, 388.

8. Plummer, A. J., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W., *Ann. N. Y. Acad. Sci.*, 1954, v59, 8.

9. Sheppard, H., Lucas, R. C., and Tsien, W. H., *Arch. Internat. de Pharmacodyn. et de Therap.*, 1955, v103, 256.

10. Sheppard, H., and Tsien, W. H., Abst. 127th Meet. of the Am. Chem. Soc., 1955, 39c.

11. Schneider, W. C., *J. Biol. Chem.*, 1948, v176, 259.

12. Ganguly, J., *Arch. Biochem. Biophys.*, 1954, v52, 186.

13. Omachi, A., Barnum, C. P., and Glick, D., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 133.

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Delaying Action of Gonadotrophins on Ovulation in the Hen.* (22059)

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The ovulation-inducing hormone (OIH) in the hen is probably the luteinizing fraction (LH)(1-3), although this action of LH independently of the follicle stimulating hormone (FSH) has not been demonstrated in either birds or mammals(4). As in other spontaneously ovulating animals such as the rat(5) and cow(6), OIH release in the hen is apparently effected over nervous pathways (7-12). The interval from OIH release (or from neural excitation for OIH release) to ovulation is estimated to be not more than about 8 hours(11-15), although it may be greater in some hens(10). A number of pharmacological agents which block LH release in the rat(5,16) were shown recently to suppress ovulation of the first (or C_1) follicle of the hen's cycle following a single injection 38 hours before expected ovulation(17). The agents included anesthetic [diallylbarbituric acid (Dial), pentobarbital Na (Nembutal), and Phenobarbital Na], anticholinergic (atropine sulfate) and antiadrenergic (SKF-501 and Dibenamine) drugs. It seemed highly improbable that some, at least, of these drugs (*e.g.*, atropine or Nembutal) could directly block the OIH release mechanism 30 hours following their injection. Suppression of ovulation was attributed instead to disruption of nervous controls over pituitary gonadotrophin secretions required for maintenance and maturation of the ovarian follicle. The prevalence of follicular atresia in hens injected 38 hours before expected C_1 ovulation and sacrificed shortly after the hour of normally expected

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ovulation(18) added weight to this conclusion. Ovulation of the second or C_2 follicle was also suppressed by the agents enumerated above, and again in certain instances, under conditions which pointed to disruption of gonadotrophin secretion for maintenance or maturation of the follicle rather than to simple "blockade" of OIH release(18 and unpublished observations).

One obvious approach to the problem thus posed was to administer gonadotrophins in the expectation that these might counteract the drug action. An anterior pituitary (AP) preparation was accordingly given intramuscularly shortly before injection of representative "blocking" agents. Contrary to expectations, the AP preparation appeared to enhance the suppressing or delaying action of the drugs. These observations led to the suspicion that the AP preparation itself might delay ovulation. This proved to be so. Conceivably, any of several hormones presumably carried by the whole AP preparation might have accounted for its delaying action. Not exhausting all these possibilities, it was nevertheless found that appropriate combinations of LH and FSH could duplicate the effects of the whole AP preparation. In a word, pituitary gonadotrophins may serve in the hen as highly effective "blocking" agents.

Materials and methods. Hens of White Leghorn and crossbred (White Leghorn x Rhode Island Red) extraction served in all but a few test groups; some Rhode Island Red hens were used in these latter groups. The battery caged birds were under artificial illumination from 6:00 a.m. through 8:00 p.m. and had access to feed and water *ad libitum.* Practically all hens were ovulating in 3-day "closed" cycles, ovulating, that is, consecutively on each of 2 days, failing to ovulate on the third day to complete one cycle, and then ovulating on the next day to initiate another cycle(11,12). Typically, the first or C_1 ovulation in the 3-day cycle occurs at about 6:00 a.m., the second or C_2 at around 10:30 a.m. of the following day. The interval between C_1 and C_2 ovulations is thus approximately 28.5 hours. Since ovulation fails to occur on the third day of the cycle, but does so at 6:00 a.m. of the day thereafter, the interval between the C_2 ovulation of one cycle and the C_1 ovulation of the succeeding cycle is some 43.5 hours. All injections were timed for effect on C_2 ovulations. Two measures of response are recognized. The first is simply the incidence of suppressed ovulations, regardless of the fate of the follicle whose ovulation was prevented. The second is the incidence of C_2 ovulations which were delayed only from the normally expected hour of one day (ca. 10:30 a.m.) until early morning hours (ca. 6:00 a.m.) of the following day. In this type of delay the follicle whose ovulation was prevented subsequently behaves like the C_1 follicle of the naturally occurring closed cycle. Such delayed ovulations are therefore designated "C_1 lapses", or merely "lapses". The induced C_1 lapse is identical with the "1-day delay" in ovulation noted elsewhere(11), and is a special case of the "C_1" type" of delayed ovulation first recognized by Rothchild and Fraps(19). Theoretically, the C_1 lapse can be induced only if the specific follicle whose ovulation was prevented is also the follicle subsequently to ovulate. Autopsy findings confirmed this important expectation. The appearance of the C_1 lapse may therefore be accepted as evidence that normally expected OIH release was prevented or failed to occur despite the presence of an ovulable follicle. The occurrence of normal ovulation, of the C_1 lapse, or of longer delays in ovulation was established by palpation procedures described earlier(11,20). Percentages of suppressed ovulations or of C_1 lapses appearing in the Tables are based on the total number of injected hens in each group. Despite care in selection of the birds, an occasional hen will fail to ovulate as expected. A very low incidence of suppressed ovulations or of C_1 lapses ($<$10 or 20%) is therefore of little or no significance. The "L/S ratio," expressed in percent, gives the proportion of C_1 lapses included in the total of suppressed ovulations. Since follicular atresia is ruled out in the C_1 lapse, but not necessarily in the case of ovulations delayed for greater intervals (2 days or more), the L/S ratio serves as an index of relative follicular immunity to indicated experimental procedures. The "blocking" agents were injected into the muscles of the

TABLE I. Effect of Male Chicken Anterior Pituitary (AP) on Incidence of Delayed C_2 Ovulations following Administration (4:00-5:00 P.M.) of Several "Blocking" Agents.

Injected agents	No. hens inj.	Suppressed ovulations No.	Suppressed ovulations %	C_1 lapses No.	C_1 lapses %	L/S ratio (as %)
Atropine	12	10	83	5	42	50
AP + atropine	11	10	91	8	73	80
Nembutal	15	8	53	5	33	63
AP + nembutal	9	8	89	8	89	100
Phenobarbital	11	10	91	1	9	10
AP + phenobarbital	11	11	100	2	18	18

leg at the following dosages: atropine sulfate, 80 mg/kg body weight, Nembutal, 60 mg/kg, and phenobarbital sodium, 100 mg/kg. The drugs were taken up in distilled water immediately before use, one ml water carrying the mg/kg equivalent. Some deaths resulted from the intramuscular injection of Nembutal at the 60 mg/kg level, although none had been encountered when the same dosage was given subcutaneously in other experiments(17).

The AP preparation consisted of anterior pituitary glands from adult Rhode Island Red male chickens. Upon removal, the glands were crushed between glass plates, air dried and subsequently powdered. The powder was suspended in distilled water, 2 or 4 mg/ml, usually shortly before injection into the pectoral muscles; a few suspensions were carried (under refrigeration) over several days. When used with "blocking" agents, the AP preparation was injected some 30-40 minutes before administration of the drugs. The LH and FSH fractions were prepared from anterior pituitary glands of sexually mature male chickens by procedures previously described in the fractionation of sheep pituitary tissue (21). Shortly before injection the fractions were dissolved, separately or together, in distilled water in concentrations which required the administration of not over 1 ml/hen. The solutions were injected into the pectoral muscles. Progesterone was dissolved in corn oil (Mazola), 5 mg/ml, and was injected into the pectoral muscles, 1 mg/hen.

Results. i. Effects of AP on drug action. Examples of drug-induced suppression of C_2 ovulation and AP effects on the drugs are given in Table I. Atropine alone or phenobarbital alone suppressed a high proportion of expected ovulations, yet AP appeared to intensify—however slightly—the suppressing action of the drugs. Nembutal alone suppressed only 53% of expected ovulations, leaving a wider margin for display of any enhancing action by AP. Significantly, AP with the drug increased the incidence of suppressed ovulations from 53 to 89%. The incidence of C_1 lapses increased appreciably when AP was used with atropine (from 42 to 73%) or with Nembutal (from 33 to 89%). With phenobarbital the AP effect was negligible, though in the direction of increased incidence. A high proportion of ovulations suppressed by AP + atropine subsequently exhibited C_1 lapses (L/S = 80%), and all ovulations suppressed by AP + Nembutal did so.

ii. The effects of AP alone. Preliminary tests with AP at several dosage levels indicated that 2 mg/hen, injected at about 4:00 p.m., yielded a fairly high incidence of sup-

TABLE II. Incidence of Suppressed Ovulations and C_1 Lapses following Intramuscular Injection of AP, 2 mg/Hen, at Differing Times before Expected C_2 Ovulation.

Time of inj. (p.m.)	Inj. to ovulation (hr)	No. hens inj.	Suppressed ovulations No.	Suppressed ovulations %	C_1 lapses No.	C_1 lapses %	L/S ratio (as %)
1:30	24	7	5	71	4	57	80
4:30	18	22	20	91	18	82	90
6:30	16	12	8	67	4	33	50
11:30	11	7	0	—	—	—	—

TABLE III. Incidence of Delayed C_2 Ovulations following Intramuscular Injection (ca. 5:00 P.M.) of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Combinations of the Fractions.

Quantities LH, mg	Quantities FSH, mg	No. hens inj.	Suppressed ovulations No.	Suppressed ovulations %	C_1 lapses No.	C_1 lapses %	L/S ratio (as %)
.005	—	11	2	18	2	18	100
.01	—	11	4	36	2	18	50
.02	—	12	7	58	4	33	57
.04	—	14	6	43	5	36	83
—	1.0	9	1	11	0	—	—
—	2.0	21	7	33	6	29	86
.02	.5	14	8	57	7	50	88
.02	1.0	27	21	78	16	59	76
.02	2.0	14	13	93	13	93	100

pressed ovulations. This level was used in all experiments summarized in Table II, which in part were carried out to ascertain variation of effect with hour of injection. Despite the relatively small number of hens injected at 1:30 p. m., the results leave little doubt that AP effectively suppressed ovulation when administered as much as 21 hours before the expected event. Four of the 7 treated hens (57%) exhibited C_1 lapses, or 80% of the 5 in which ovulation was suppressed. Following 4:30 p. m. injections, ovulation was suppressed in 20 out of 22 hens. Eighteen of the 22 injected hens (82%), or 90% of the 20 in which ovulation was suppressed, exhibited C_1 lapses. Ovulation was suppressed in 67% of 12 hens injected at 6:30 p. m., but C_1 lapses occurred in only 4 or 33%. As was anticipated from earlier experience, injection of AP at 11:30 p.m. failed to delay ovulation in any of 7 hens.

iii. Effects of LH, FSH and LH + FSH. As can be seen in Table III, LH suppressed few ovulations at the 0.005 mg level, and no large proportion at any of the dosages administered. The incidence of C_1 lapses was relatively low at the 0.02 mg level (33%), which suppressed ovulation in 58% of 12 injected hens. At the 0.04 mg level the proportion of suppressed ovulations dropped somewhat, although the incidence of C_1 lapses increased slightly; the L/S ratio increased from 57 to 83%. Administration of FSH alone at the 1.0 mg level was without appreciable effect in suppressing ovulation. The moderate incidence of suppressed ovulations following injection of FSH at the 2 mg level might possibly be attributed to contaminating LH, and is discussed later. In combining LH and FSH, LH at the 0.02 mg level was injected with increasing levels of FSH (Table III). The incidence both of suppressed ovulations and of C_1 lapses is seen to increase with increasing FSH levels, though at the 1 mg level of FSH the proportion of C_1 lapses did not keep pace with that of suppressed ovulations. This was reflected in the L/S ratio, which came to 76%. The combination of 0.02 mg LH + 2 mg FSH suppressed ovulation in 13 of 14 injected hens, and all of these exhibited the C_1 lapse.

iv. Progesterone effects. Twenty hens were injected with AP (2 mg/hen) at about 4:30 p. m. for delay of C_2 ovulation. The same hens received progesterone (1 mg/hen, intramuscularly) at about 2:30 a. m. of the following morning, *i.e.*, at about the hour of normally expected OIH release. Fifteen of the 20 hens (75%) ovulated either normally or in response to progesterone. This result is to be compared with the effect of AP alone (4:30 p. m. injections, Table II); here only 2 of 22 hens (9%) ovulated at the normally expected hour. It is to be noted that the incidence of progesterone-induced ovulations in AP treated hens is of the same order as was the incidence of C_1 lapses following the administration of AP alone.

Discussion. When injected intravenously, 0.001 mg/hen, 14 hours before expected C_1 ovulation, the same LH fraction used in the present experiments induced ovulation prematurely in some 50% of treated birds(3). The

FSH fraction was about equally effective at the 0.5 mg/hen level. This quantity of FSH (500 times the LH dose) presumably carried sufficient LH as a contaminant to account for the induced ovulations. The moderate effectiveness of FSH in delaying C_2 ovulation (Table III, 2 mg/hen) probably represents, in part at least, the action of LH. If this is so, we may conclude that FSH in itself has little or no capacity to delay C_2 ovulation, but that it substantially increases the potency of LH in this respect. The increased effectiveness of LH in combination with FSH observed here is apparently another instance of the augmentation effect so often encountered when the two fractions are combined(22,23).

Incomplete or partial stimulation of the ovulable follicle by subovulatory doses of LH may result in atresia(24) and thus "suppress" its normally expected ovulation. Van Tienhoven(25) has shown recently that interruption of progesterone-induced OIH release may likewise cause atresia of the ovulable follicle. Interruption of normal OIH release would presumably have the same effect. In the present experiments, atresia of the ovulable follicle might have occurred (though not necessarily), in any hen which failed to exhibit the C_1 lapse. Under appropriate conditions, however, gonadotrophins are capable of inducing the C_1 lapse in practically all injected birds. It is accordingly this characteristic effect which calls for examination and, if possible, explanation.

A first question concerns the immediate source of the suppressed ovulation appearing in the C_1 lapse. The fact that the suppressed C_2 ovulation is typically delayed only until the approximate hour of C_1 ovulation suggests failure of nervous stimulation for normally expected OIH release, such stimulation occurring at the outset of the next diurnally recurrent period of high neural sensitivity(11, 12). The response of gonadotrophin treated hens to progesterone, which causes OIH release by stimulation of the neural component of the release mechanism(7-10,12,25), confirms this inference. We may then conclude that the gonadotrophin-induced C_1 lapse makes its appearance in consequence of a transient "blockade" of the nervous activity required for OIH release.

There are no grounds for supposing that gonadotrophins might act directly as "blocking" agents (*e.g.*, like central depressants). On the contrary, we should expect them to act directly and perhaps exclusively upon the ovary and its secretory functions(4). The causes of gonadotrophin-induced lapses must then be sought in modified concentrations of circulating hormones of ovarian origin.

In appropriate combinations, LH and FSH would be expected to favor estrogen production in the hen, as they certainly do in the rat(26,27). Although estrogen may cause ovulation in the rat and other polyestrous mammals(4,28 for references), it delays ovulation in both the ring dove(29) and the hen (11). The proportion of C_2 ovulations suppressed by estradiol benzoate was only moderate in the hen (about 50%), but some 90% of these subsequently exhibited the C_1 lapse. Moreover, estrogen apparently induced C_1 lapses by increasing threshold requirements in the neural component of the OIH release mechanism(11). In all important respects, then, estrogen and gonadotrophin effects are similar. The gonadotrophin-induced lapse may thus have its origin, in part at least, in elevated levels of circulating estrogen.

Despite the similarity of qualitative actions, gonadotrophins appear to be more effective than either estradiol benzoate or diethylstilbestrol in delaying C_2 ovulation. This may mean that the endogenous estrogen is more "potent" than are the injected substances, or that it is secreted in a fashion not reproduced by injection. There remains, however, another possible aspect of endogenous estrogen production which may not be encountered when estrogen is injected. The gonadotrophin enforced elaboration of estrogen may be associated with delayed or decreased production by the ovary or the maturing follicle of the hormone—possibly a progestin(11,12)—believed to stimulate the neural component of the OIH release mechanism. In this event, gonadotrophin-induced lapses may stem in part from subthreshold levels of the postulated circulating excitation hormone.

If exogenous gonadotrophins result in an altered balance of ovarian hormones (estrogen

and progestin), it is not impossible that the naturally occurring OIH release—whether this consist of FSH and LH or LH alone—may do the same. Such a recurrent periodicity in ovarian hormone levels might well play an important part in the determination of lag relationships, length of cycle, and time of onset of the period of lapse(11,12). There is little point in carrying these speculations further at this writing, but they may serve to underscore the very real need for identification of the sex steroids of the ovulating hen and determination of varying concentrations with reference to critical events in the cycle.

The experiments described in this paper do not tell us much about the manner in which the usual nervous "blocking" agents delay ovulation when the delay (or suppression) cannot be attributed to direct blockade of the neural component of the OIH release mechanism(17,18). The increased proportions of C_1 lapses effected by the AP preparations in Nembutal or atropine treated hens may well signify. however, some amelioration of drug action by gonadotrophins or a gonadotrophin Experiments looking toward a clarification of these relationships are in progress.

Summary. Antiadrenergic, anticholinergic or central depressant drugs suppress or delay, in varying proportions, ovulation of the second or C_2 follicle of the hen's sequence following their administration some 18-20 hours before normally expected ovulation. In an attempt to counteract these effects, a whole anterior pituitary (AP) preparation from cocks was injected intramuscularly shortly before injection of several of the drugs. The incidence of suppressed ovulations was increased by the AP preparation, and increased proportions of suppressed ovulations exhibited C_1 lapses, *i.e.*, the normally expected C_2 ovulation was delayed only until the hour of typical C_1 ovulation on the day following its suppression. Intramuscularly injected 18-19 hours before expected C_2 ovulation, the AP preparation alone suppressed ovulation in most hens, with a high proportion of C_1 lapses. Under similar conditions, luteinizing hormone (LH) or follicle stimulating hormone (FSH) from cock pituitaries suppressed ovulation in some hens. Administration of 0.02 mg LH + 2 mg FSH/hen suppressed ovulation in 93% of injected birds, and all of these exhibited the C_1 lapse. Progesterone injected at about the hour of normally expected release of ovulation-inducing hormone (OIH) effected ovulation in most hens previously treated with the AP preparation, indicating that gonadotrophin-induced lapses resulted from a transient "blockade" of the nervous mechanism controlling OIH release. Since estrogens similarly "block" C_2 ovulation in the hen, the gonadotrophin-induced delay may be attributed to increased levels of circulating estrogen. Enforced estrogen production may however be associated also with reduced or delayed output of the hormone believed to excite the nervous component of the OIH release mechanism.

1. Fraps, R. M., Olsen, M. W., and Neher, B. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 308.

2. Fraps, R. M., and Dury, A., *Anat. Rec.*, 1943, v87, 442.

3. Fraps, R. M., Fevold, H. L., and Neher, B. H., *ibid.*, 1947, v99, 571.

4. Hisaw, F. L., *Physiol. Rev.*, 1947, v27, 95.

5. Everett, J. W., *Ciba Found. Coll. Endocrinol.*, 1952, v4, 167.

6. Hansel, W., and Trimberger, G. W., *J. Animal Sci.*, 1951, v10, 719.

7. Fraps, R. M., and Case, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 167.

8. Zarrow, M. X., and Bastian, J. W., *ibid.*, 1953, v84, 457.

9. Huston, T. M., and Nalbandov, A. V., *Endocrinology*, 1953, v52, 149.

10. Van Tienhoven, A., Nalbandov, A. V., and Norton, H. W., *ibid.*, 1954, v54, 605.

11. Fraps, R. M., *Proc. Nat. Acad. Sci.*, 1954, v40, 348.

12. ———, *Mem. Soc. Endocrinol.*, No. 4, in press.

13. Fraps, R. M., Riley, G. M., and Olsen, M. W., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 313.

14. Rothchild, I., and Fraps, R. M., *Endocrinology*, 1949, v44, 134.

15. ———, *ibid.*, 1949, v44, 141.

16. Everett, J. W., and Sawyer, C. H., *ibid.*, 1950, v47, 198.

17. Fraps, R. M., and Conner, M. H., *Nature*, 1954, v174, 1148.

18. ———, *Anat. Rec.*, 1955, v121, 295.

19. Rothchild, I., and Fraps, R. M., *Endocrinology*, 1947, v40, 55.

20. Fraps, R. M., and Dury, A., PROC. SOC. EXP.

BIOL. AND MED., 1943, v52, 346.
21. Fevold, H. L., *Annals N. Y. Acad. Sci.*, 1943, v43, 321.
22. Fevold, H. L., Hisaw, F. L., and Leonard, S. L., *Am. J. Physiol.*, 1931, v97, 291.
23. Fevold, H. L., and Hisaw, F. L., *ibid.*, 1934, v109, 655.
24. Fraps, R. M., and Dury, A., *Anat. Rec.*, 1942, v84, 521.
25. Van Tienhoven, A., *Endocrinology*, 1955, v56, 667.
26. Fevold, H. L., *ibid.*, 1941, v28, 33.
27. Greep, R. O., van Dyke, H. B., and Chow, B. F., *Endocrinology*, 1942, v30, 635.
28. Sawyer, C. H., Everett, J. W., and Markee, J. E., *ibid.*, 1949, v44, 218.
29. Dunham, H. H., and Riddle, O., *Physiol. Zool.*, 1942, v15, 383.

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Effect of Temperature and Humidity on Nasal Flora of Mice. (22060)

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The possibility that atmospheric conditions are reflected in changes in physiological status of the nose and nasopharynx which are related to susceptibility to upper respiratory infections and poliomyelitis have been suggested. Thus, Armstrong(1) observed a correlation between absolute humidity and incidence of poliomyelitis in certain localities and suggested that atmospheric humidity influences susceptibility by its action on the nasopharynx.

In the present investigation an attempt was made to demonstrate local alterations in normal microflora of nasal passages of mice after exposure to different atmospheres of temperature and humidity, preliminary to a study of association of these changes with local resistance.

Material and methods. White Swiss male mice (9-12 g) were used. Atmospheres of low and high humidity were prepared in metal chambers with glass covers in which cages containing the mice were placed. A low relative humidity in range of 25-30% was maintained by introducing dry air into an enclosed chamber and measured directly by means of hydrodial supplied by the Bendix Co. of Baltimore, Md. A high relative humidity was maintained by introducing air saturated with water into the enclosed chamber. Three sets of the experimental chambers were maintained at 21°C, to study the effect of humidity and normal temperatures. One set was maintained at approximately 31°C to study the effect of increased temperature and humidity. Mice were kept at 21°C with a relative humidity of 45-50% for at least 2 to 3 weeks before they were subjected to experimental conditions. They were fed a balanced diet, and every care taken to avoid infection, exposure, or overcrowding. Bacterial flora of the nasal tract was determined by plating nasal washings on blood agar plates. Nasal washings were obtained as follows: Mice were killed by ether. The skin covering anterior part of head was removed after being moistened with ethyl alcohol. The nasal bone covering the nasal passage was lifted using aseptic technic, thus exposing the nasal cavity. Using a capillary pipette, .05 ml of sterile buffered saline was introduced and after drawing it up and down several times, the washing was spread on a blood agar plate using sterile bent glass rod. The plates were incubated at 37°C for 48 hours and the number and type of normal flora were determined. Nasal washings from 20 mice which had been exposed to low humidity were tested for antibacterial action on isolated gram positive and gram negative bacteria.

Results. The effect of temperature and humidity on normal flora of nasal passage was studied by exposing 4 groups of mice to conditions mentioned previously for 3 weeks. The results summarized in Table I indicated

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TABLE I. Effect of Temperature and Humidity on Normal Flora of the Nasal Tract of Mice.

Humidity	50%		25%		85-90%		85-90%	
Temp.	21°C		21°C		21°C		31°C	
	Total No.	% gram positive	Total No.	% gram positive	Total No.	% gram positive	Total No.	% gram positive
	>1000	50	3	66	>1000	90	about 1000	70
	"	90	111	5	"	90	"	75
	"	90	5	40	about 1000	95	200	90
	"	90	36	22	>1000	95	>1000	90
	450	70	102	2	"	90	"	60
	1000	50	33	9	"	95	700	70
	"	65	69	6	"	90	>1000	70
	"	84	45	28	"	90	500	80
	250	60	47	28	about 1000	90	500	50
	>1000	80	95	5	"	90	about 1000	90

a significant reduction in total numbers of bacteria in the nasal tract of mice kept under low humidity (25%) as compared to those kept at normal (50%) or high (85-90%) humidity. In none of the mice kept at low humidity did the numbers of bacteria equal the lowest number observed in any of the mice kept under normal conditions.

At normal or high humidity the flora was predominantly gram positive, whereas under low humidity the reverse was true. This appears to be due to the marked reduction in gram positive flora and whatever the mechanism involved in these changes is, it seems to have its effect primarily in this group. This inhibition was maximum after 3 weeks exposure. Fig. 1 shows typical plates of normal flora of mice under these different conditions. Fig. 2 shows typical plates of normal flora in nasal washings of mice exposed to low humidity for one and two weeks time as compared with organisms obtained from mice under normal conditions. No antibacterial action could be demonstrated in nasal washings by filter disc method.

Discussion. Our results suggest a significant change in the physiological condition of the nasal passage in response to changes in humidity. Changes in temperature did not produce similar effects. However, there were indications that mice kept under high temperature were "stressed" in that systemic responses associated with stress phenomenon could be demonstrated(2). Previous work on correlation of temperature changes with upper respiratory infections has been concerned

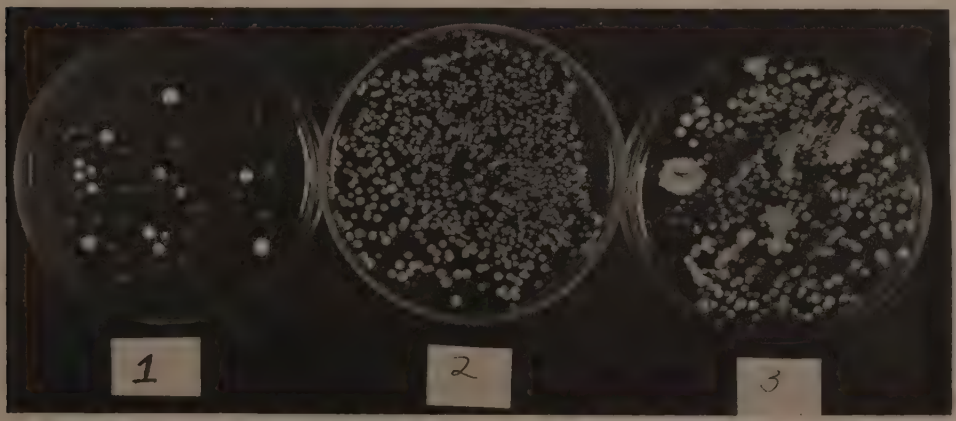

FIG. 1. Plates of nasal washings of mice exposed to the following conditions: 1. Normal temperature (21°C) and low humidity (25%). 2. *Idem*, but high humidity (90%). 3. High temperature (31°C) and high humidity (90%).

FIG. 2. Plates of nasal washings of mice exposed to normal temperature (21°C) and low humidity (25%) for different periods of time. 4. Control (21°C, 50%). 5. Mice exposed for one week. 6. Mice exposed for 2 weeks.

with the effect of chilling on the physiology of the nasopharynx(3).

Specific antibodies in nasopharyngeal secretions have been demonstrated. Amoss and Taylor(4), Howitt(5), and Bell(6) found specific antibodies against poliomyelitis virus in nasal secretions of a portion of persons examined. Burnet, Lush, and Jackson(7) reported a heat-labile, possibly enzymic substance, in nasal secretions which inactivated certain viruses, notably those of influenza, herpes, and poliomyelitis, but not vaccinia virus. Our failure to show any antibacterial substance in the nasal washings from mice kept at low humidity may be due to the fact that such a substance was present in quantities too minute to be observed by the method used.

Preliminary results have indicated further that conditions favorable for gram positive bacteria in nasal passages appeared to be favorable for infection with influenza virus(2). The reason for the relationship is not clear. It may be that the effect of high humidity on the mucous membrane produced a more favorable environment for gram positive organisms, which in turn affected the mucous membranes in such a way as to make them more susceptible to infection by the virus. On the other hand, it may be that the relationship is more direct and that the change favorable for gram positive organisms also favored the virus. Further work is needed to investigate the association of changes in normal flora of the nasal passage and lowering of local resistance.

Summary. The count of the normal bacterial flora of the nasal cavity was markedly reduced when mice were maintained under low humidity for three weeks, whereas no significant change was observed under high humidity. The reduction was noted mainly in the gram positive organisms.

1. Armstrong, C., *Am. J. Pub. Health*, 1950, v40, 1296.

2. Mayyasi, S. A., 1953, to be published.

3. Mudd, S., and Grant S., *J. Exp. Med.*, 1921, v34, 11.

4. Amoss, H., and Taylor, H., *ibid.*, 1917, v25, 507.

5. Howitt, B. F., *J. Infect. Dis.*, 1937, v60, 113.

6. Bell, E. J., *Am. J. Hyg.*, 1943, v47, 351.

7. Burnet, F. M., Lush, D., and Jackson, A. V., *Brit. J. Exp. Path.*, 1939, v20, 377.

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Concentration and Distribution of Cholesterol in Muscle and Adipose Tissue.* (22061)

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It is widely assumed that most or all of the cholesterol in muscles, for example in meats, is associated with adipose tissue and interstitial fat and that, in general, with increasing fatness there is an increasing cholesterol content of the whole muscle. This view is reflected in common dietetic practice in which fatty meats are proscribed in low-cholesterol diets, though lean, fat-trimmed meats are allowed.

Actually, however, data have not been available on the distribution of cholesterol between the muscle proper and the associated fat. The present paper summarizes a study on this question with muscle and adipose tissue of 3 species of meat animals, of rabbits, of chickens and of man.

Materials and methods. Beef, veal, pork, suckling pig, mutton, lamb and chickens were purchased fresh in Minneapolis markets. Rabbits were obtained from the University Medical School. Fresh human tissues (abdominal wall muscle and associated adipose tissue and psoas muscle) were provided by the Department of Pathology of the University of Minnesota Medical School. Both commercial and choice grades of round and rib steaks of beef were studied. Leg and loin cuts of veal, mutton and lamb and the longissimus dorsi muscles of rabbit were used. Light (breast) and dark (thigh) meats from roosters, hens and chicks were separately studied. Subcutaneous fat of rabbits, hens and roosters were also analyzed. For each sample the adipose tissue and gross interstitial fat were separated from the lean as completely as possible by careful scalpel dissection. The separated portions were minced in a meat grinder and stored in well-sealed containers at –20°C before analysis. Moisture was determined by drying at low temperature in a vacuum desiccator(1). "Fat" was measured as the weight of the dried ether extract obtained by prolonged extraction in the Soxhlet apparatus. For cholesterol the procedure of Pihl(2) was followed until the chloroform extract was obtained. This was then evaporated, the residue was dissolved in acetone-alcohol, and the cholesterol was precipitated and measured as the digitonide according to the method of Foldes and Wilson(3). Tests of the method gave 98 to 104% recovery of added cholesterol. All measurements were made in duplicate on each sample.

Results. There were no significant differences in the cholesterol results for the different grades and cuts of meat so these are not differentiated in Table I which summarizes 8 independent samples of beef, 4 each of lamb and pork and 2 each for the other

TABLE I. Results from Analyses on Meat. EE = ether extract; cholesterol, mg % EE = mg of cholesterol/100 g of ether extract. Mean values and ± stand. deviations (beef, pork, lamb) or half the range (veal, pig, mutton, rabbit).

	Dissected muscle			Adipose tissue			
						Cholesterol	
Item	EE, %	Water, %	Chol., mg %	EE, %	Water, %	mg %	mg % EE
Beef	5.5 ± 2.3	72.6 ± 3.0	46.8 ± 4.7	75.7 ±10.9	21.6 ± 9.7	81.5 ±18.9	104.6 ±30.0
Veal	1.6 ± .4	74.5 ± 0	71.9 ± 1.0	85.5 ± 3.6	13.6 ± 3.9	76.4 ± 4.3	89.2 ± 1.0
Pork	5.0 ± 1.0	71.0 ± .7	46.4 ±10.1	90.0 ± 3.9	7.9 ± 3.0	72.6 ±11.2	80.2 ±14.8
Pig	3.0 ± .5	76.4 ± .9	83.7 ± .6	68.5 ± 4.3	24.9 ± 3.1	89.3 ±10.0	121.6 ±22.4
Mutton	3.0 ± .5	73.7 ± .1	51.8 ± .8	90.2 ± 1.5	6.6 ± 1.2	76.3 ± 6.0	84.4 ± 5.3
Lamb	5.3 ± 2.4	70.6 ± 1.1	60.9 ± 1.3	92.7 ± 4.9	4.7 ± 3.6	67.4 ±20.0	73.7 ±26.8
Rabbit	.9 ± .3	74.5 ± 2.8	43.5 ± 1.0	89.2 ± 0	8.5 ± .7	133.7 ±47.6	149.8 ±53.3

* This work was made possible by a fellowship grant from Swift and Co., Chicago.

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TABLE II. Results from Analyses on 5 Patients. Column headings as in Table I.

			Dissected muscle			Adipose tissue			
Case	Age	Sex	EE, %	Water, %	Chol., mg %	EE, %	Water, %	Cholesterol mg %	Cholesterol mg % EE
BS	43	♀	3.4	77.4	65.8	55.5	28.7	150.2	270.5
M	65	♀	22.0	58.9	112.7	96.9	2.3	189.8	195.9
AJ	67	♂	8.3	71.6	138.4	87.4	11.0	323.1	396.6
LB	57	♂	5.6	74.3	59.2	88.4	10.5	95.4	107.9
CM	7	♀	6.6	71.8	64.3	93.9	—	101.1	107.7

items. Table II gives the data from muscle and adipose tissues from 4 adults and a 7-year-old girl. Subjects M and CM had brain tumors, subject BS had carcinoma of the breast, subject AJ had carcinoma of the pancreas, and subject LB had rheumatic heart disease. Table III summarizes the data from chickens. It was impossible to obtain enough adipose tissue from the chick for separate analysis. Inspection of Tables I, II and III shows that, in general, the concentration of cholesterol in the ether extract of the dissected muscle is much higher than in the ether extract of the adipose tissue. However, some of the ether extract obtained from the dissected muscles represents adipose tissue not removed by dissection so some allowance must be made for this. The concentration of cholesterol in such residual adipose tissue should be the same, per unit of ether extract, as in the adipose tissue actually dissected out and analyzed so it should be possible to calculate the amount of cholesterol in the muscle associated with this adipose tissue.

Let E and e represent the grams of ether extract obtained from 100 g of adipose tissue and dissected muscle, respectively, and let K and k be the grams of cholesterol contained in these. Further, let R be the grams of ether extract in 100 g of dissected muscle that is attributable to adipose tissue residue in that muscle, and let C be the grams of cholesterol per 100 g of dissected muscle that is not attributable to residual adipose tissue. Then: 1), $e = R + C$, and 2), $(k-C)/R = K/E$, so 3), $C = (Ek-Ke)/(E-K)$.

Computations from the data in Tables I, II and III are summarized in Table IV. Very little of the cholesterol in the dissected muscle is attributable to residual adipose tissue like that dissected away and it is indicated that truly "lean" muscle contains a very significant amount of cholesterol in all animals, including man.

Further inspection of Table IV shows that this non-adipose tissue cholesterol tends to be higher in the young than in the mature animals of the same species (beef vs. veal, pork vs. pig, mutton vs. lamb). The data do not allow exact calculations for the chicken but in this case, too, it would appear that the rule must hold (Table III). The data from the human subjects are inadequate for the examination of this question and, in any case, may be unrepresentative of normal persons.

Discussion. In most of the previous studies of cholesterol in tissues the Liebermann-Burchard reagent has been applied directly to the non-saponifiable residue from the lipid extraction without separating the digitonide. In

TABLE III. Results from Analyses on Roosters, Hens, and Chicks. L = light meat, D = dark meat. Column headings as in Table I.

		Dissected muscle			Adipose tissue			
Item		EE, %	Water, %	Chol., mg %	EE, %	Water, %	Cholesterol mg %	Cholesterol mg % EE
Rooster	L	.6	72.6	31.7	97.5	4.0	76.4	78.4
"	D	.8	73.1	55.3				
Hen	L	1.5	70.3	31.7	97.3	4.6	91.3	93.9
"	D	4.9	71.1	53.7				
Chick	L	.6	74.8	60.1	—	—	—	—
"	D	4.5	77.3	97.6	—			—

TABLE IV. Computed Mean Values for Concentration of Cholesterol in mg per 100 g of Muscle Free of Adipose Tissue.

	Cholesterol		Cholesterol
Patient BS	58.8	Beef	43.3
" M	89.4	Veal	71.6
" AJ	117.8	Pork	44.6
" LB	56.4	Pig	82.4
" CM	61.3	Mutton	50.7
Rooster L	31.5	Lamb	60.3
" D	55.1	Rabbit	42.6
Hen L	30.8		
" D	51.6		

the present study this "direct" proceeding was applied in parallel with our digitonin method in aliquots from 34 samples. The "direct" result averaged 16.9% higher (s.d. $= \pm 10.8\%$) than the digitonin result so it appears that about a sixth of the non-saponifiable Liebermann-Burchard reacting material in muscle is not cholesterol. This explains, in part, the fact that most of the previously published analyses for cholesterol in meats are higher than those given here(cf, 4-6).

It has been reported that different muscles in the same animal may differ somewhat in cholesterol concentration(7-9), and it has been suggested that these differences are related to differences in the customary activity of the muscles(10-12). It is interesting that red muscles (semi-tendinosus) contain more cholesterol than white (biceps femoralis) muscle in the rabbit(8) and that, as reported here, the same rule holds in the chicken(7).

If to these considerations we add the fact of higher concentrations of cholesterol in the muscle tissue of younger animals it would appear that perhaps there is a general rule for the cholesterol concentration in muscle to be related to the total energy metabolism representing growth of the muscle and muscular work, or both. And it is difficult to avoid the conclusion that, far from being inert, cholesterol has an important role in structure or function of the muscle.

The present calculations for the non-adipose tissue cholesterol in muscle are approximations, of course, because there are certainly other ether-soluble materials in muscle besides cholesterol that are not associated with adipose tissue. Equation 1), above, more properly should be written 4), $e = R + C + X$, where X represents other ether-soluble materials not accounted for by adipose tissue. Presumably, X might include some of the lecithin, for example, found in muscle. But X must be a small quantity compared to R and the error in the estimation of C resulting from its neglect should be trivial.

Summary. 1. Analyses are reported for cholesterol contents of muscle and adipose tissue from beef, veal, pork, pig, mutton, lamb, rabbit, chicken and man. It is shown that a large proportion of the cholesterol in muscle is not attributable to residual adipose tissue or any tissue of similar composition. 2. Concentration of cholesterol in muscle, apart from associated adipose tissue, is of the order of 40 to 50 mg per 100 g in the adult mammals studied and in the dark meat of chickens. Higher values were found in two elderly human beings. The corresponding cholesterol concentration in the white meat of chickens is of the order of 30 mg per 100 g. 3. There is a general tendency for muscle cholesterol concentration to be considerably higher in young, growing animals than in adults of the same species. This reinforces the theory that most of the cholesterol in muscle has a definite metabolic or structural function.

1. Winton, A. L., and Winton, K. B., *The Analysis of Foods*, John Wiley and Sons, New York, 1945, 59.

2. Pihl, A., *Scand. J. Clin. Lab. Invest.*, 1952, v4, 115.

3. Foldes, F. F., and Wilson, B. C., *Anal. Chem.*, 1950, v22, 1210.

4. Kelsey, F. E., *J. Biol. Chem.*, 1939, v127, 15.

5. Okey, R., *J. Am. Diet. Assn.*, 1945, v21, 341.

6. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., *Arch. Bioch.*, 1943, v3, 203.

7. Lawaczeck, H., *Z. physiol. Chem.*, 1923, v125, 229.

8. Embden, G., and Lawaczek, H., *ibid.*, 1923, v125, 199.

9. Bloor, W. R., *J. Biol. Chem.*, 1936, v114, 639.

10. Bloor, W. R., and Snider, R. H., *ibid.*, 1934, v107, 459.

11. Bloor, W. R., *ibid.*, 1937, v119, 451.

12. ———, *ibid.*, 1940, v132, 77.

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Cardiac, Renal and Hepatic Hypertrophy in Pyridoxine-Deficient Rats.* (22062)

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A statistically significant increase in the weight of the heart and kidneys of the pyridoxine-deficient rat was reported by Agnew (1,2). Olsen and Martindale(3) recently confirmed this work and also reported an increase in the weight of the liver of the pyridoxine-deficient rat.

The present study was undertaken to see: (1) if the increases in weight of the heart, kidneys, and liver could be found after feeding the deficient diet for a much shorter period of time than in previous experiments; and (2) if these organs were truly hypertrophied or merely heavier by virtue of increased tissue fluid content.

Methods. Male Wistar weanling rats, obtained from the Charles River Breeding Laboratories, Boston, were used, and were housed in individual cages with wide wire-screened bases. The synthetic diet used consisted of sucrose 72%, casein (Sheffield "vitamin-free") 18%, corn oil (Mazola) 5%, salt mixture(4) 4%, cod liver oil 1%. Choline chloride (1.5 g/kilo) was added, and then appropriate amounts of the following substances in alcoholic solution or suspension to give the following concentrations (mg/kilo) in the complete diet: calcium pantothenate 20, niacin 20, riboflavin 3, thiamin 3, pyridoxine HCl (controls only) 3, folic acid 1, biotin 0.2. Fresh diet was made up weekly and stored in the refrigerator. The animals were weighed daily, and had unlimited access to water. The design of the experiment was similar to that used in previous work(1), and involved the use of 12 trios of rats, each trio (littermates) being arranged thus: Rat 1 received the experimental diet *ad lib.* with all vitamin supplements; 2 received the diet *ad lib.* with all vitamin supplements except pyridoxine; No. 3 was paired-weighed with No. 2 but was given pyridoxine; *i.e.* it received the amount of diet required to keep it equal in weight to No. 2. After 55 days the animals were anesthetized with ether, bled by severance of neck vessels, and the livers, kidneys and hearts dissected out; the hearts were sliced open and any blood removed from the chambers. The organs were washed in 0.9% NaCl solution, gently dried between sheets of filter paper, and the wet weights determined on a Roller-Smith Precision balance. The organs were placed on watch glasses, dried at 58°-60°C for 7 days, and the dry weights then determined.

Results. The results are summarized in Table I. The wet and dry weights of the hearts and kidneys of the pyridoxine-deficient animals were significantly heavier (g/100 g bodyweight) than those of the *ad lib.* and paired-weighed controls. The wet and dry weights of the livers of the pyridoxine-deficient rats, although significantly heavier than those of the paired-weighed controls, did not differ significantly from those of the *ad lib.* controls. Thus it would appear that the increase in weight of these organs is a true hypertrophy and not the result of an increased tissue fluid content in them. Increased fat content was not a factor as histological examination of frozen sections of tissues obtained in earlier experiments(1,2) did not reveal the amount of fat that would have been expected if the increase in organ weight had been caused by increased fat; further, when the dried hearts and kidneys referred to in Table I were defatted, those that belonged to pyridoxine-deficient rats were still heavier than those of the *ad lib.* and paired-weighed controls.

The results obtained (Table I) also show that protracted feeding of the pyridoxine-deficient diet is not necessary to induce hypertrophy of the heart, kidneys and liver as

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TABLE I. Wet and Dry Organ Weights (g/100 g Body Weight) of *Ad lib.* Fed, Paired-Weighed, and Pyridoxine-Deficient Rats.

Organ	*Ad lib.* control (11 animals*)	Paired-weighed control (12 animals)	Pyridoxine-deficient (12 animals)
Body wt	310.9 ±30.6	124.7 ±9.6	130.5 ±10.1
Liver, wet	4.30 ± .66	3.34 ± .27	4.24 ± .45
dry	1.38 ± .08	1.04 ± .09	1.38 ± .18
Kidney, wet	.689 ± .045	.834 ± .069	1.159 ± .138
dry	.166 ± .008	.194 ± .014	.261 ± .030
Dried and defatted	.147 ± .007	.174 ± .012	.234 ± .026
Heart, wet	.307 ± .022	.344 ± .022	.435 ± .068
dry	.069 ± .005	.079 ± .005	.100 ± .017
Dried and defatted	.064 ± .004	.073 ± .005	.092 ± .015

* Originally 12 animals, but one was accidentally killed before experiment ended.

the duration of the experiment was only 55 days. Agnew(1) had previously reported the occurrence of marked cardiac and renal hypertrophy in rats that had been on the deficient diet for periods ranging from 79 to 175 days; and Olsen and Martindale(3) had obtained similar results after 266 days. These latter workers also reported the occurrence of liver hypertrophy in their deficient rats, but in the present experiments the weights of the livers of the pyridoxine-deficient rats, although significantly heavier than those of paired-weighed controls, did not differ significantly from those of *ad lib.* controls.

Discussion. The reason for the significant increase in the weight of such important organs as the heart, kidneys and liver in the pyridoxine-deficient rat is as yet unknown. It is possible that the increased heart weight is secondary to the mild hypertension that has been reported(5) in pyridoxine-deficient rats; also, it may be that the increased kidney weight is the result of the increased excretion of urea that has been described(6) in pyridoxine-deficient rats. Whatever the explanation, pyridoxine deficiency in the rat appears to be another method for inducing cardiac, renal and hepatic hypertrophy, and one that may prove of value in future studies of organ growth and function.

Summary. The wet and dry weights of the heart and kidneys of pyridoxine-deficient rats were significantly heavier (g/100 g body-weight) than those of *ad lib.* and paired-weighed littermate controls. The wet and dry weights of the livers of pyridoxine-deficient rats, although significantly heavier than those of paired-weighed controls, did not differ significantly from those of *ad lib.* controls. The increased weight of these organs is considered to be a true hypertrophy, and was observed after a period of 55 days. It is felt that this relatively rapid method of inducing hypertrophy of these important organs may prove a useful tool in studies of organ growth and function.

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1. Agnew, L. R. C., *Brit. J. Nutrit.*, 1949, v3, 217.
2. ———, *J. Path. Bact.*, 1951, v63, 699.
3. Olsen, N. S., and Martindale, W. E., *J. Nutrit.*, 1954, v53, 329.
4. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.
5. Olsen, N. S., and Martindale, W. E., *J. Nutrit.*, 1954, v53, 317.
6. Beaton, J. R., Beare, J. L., White, J. M., and McHenry, E. W., *J. Biol. Chem.*, 1953, v200, 715.

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Effect of Ascorbic Acid on Cysteine Requirement of Lactobacilli.* (22063)

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In a study of amino acid requirements of a collection of oral lactobacilli it was found that, in addition to other amino acids required for growth, cysteine was needed by 23 of 28 oral cultures and also by 3 of 5 American Type Culture Collection lactobacilli(1). This result was obtained with a chemically defined medium containing 18 amino acids including methionine, needed B vitamins, purines and pyrimidines, glucose, acetate, and salts. The proportion of lactobacilli requiring cysteine under these conditions was, in general, similar to the previous result of Dunn *et al.*(2) who found that 8 of 12 lactobacilli (exclusive of other lactic acid bacteria studied) needed cysteine for growth. These lactobacilli were mostly ATCC strains obtained originally from dairy products or fermentations. Other workers also have studied the needs of certain individual strains which, for the most part, were included in the study of Dunn *et al.*(2) and need not be cited in detail here.

TABLE I. Effect of Ascorbic Acid on Cysteine Requirement of Lactobacilli.

Cultures	No. of cultures growing in chemically defined medium*		
	Complete amino acid medium	On omission of cysteine	Ascorbic acid substituted for cysteine
L. casei	12	1	2†
" , lactose neg.	4	0	0
L. plantarum	7	3	3
L. acidophilus	4	0	0
L. fermenti	8	2	6
L. brevis	1	0	0
	36	6‡	11

* The chemically defined medium contained 200 mg DL-methionine and 100 mg L-cysteine • HCl per 1000 ml. The detailed composition has been given elsewhere(1).

† Slow growth of 1 of the 2 cultures.

‡ The 6 cultures which grew on omission of cysteine were: 1 oral *L. casei*, *L. plantarum* ATCC 8014 (*i.e.*, *L. arabinosus* 17-5), 2 oral *L. plantarum*, *L. fermenti* ATCC 9338, and one oral *L. fermenti*. Growth of most of these cultures was stimulated to some extent by added cysteine though cysteine was not essential for growth.

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TABLE II. Response of Certain Lactobacilli to Ascorbic Acid and Cysteine.

Culture designation and strain No.	Additions to medium*		Growth,† days			
	Ascorbic acid, .3 mg/ml	Cysteine • HCl, 100 μg/ml	2	3	4	5-7
L. casei 1006	0	0	3	8	11	13
	0	+	78	83		
	+	0	15	27	49	51
	+	+	62	82		
L. fermenti 55	0	0	1	2	2	1
	0	+	86	88		
	+	0	73	87		
	+	+	86	89		
" 1007	0	0	2	2	1	2
	0	+	4	5	6	76
	+	0	11	54	66	
	+	+	9	17		63
L. casei 1009‡ (lactose neg.)	0	0	11		14	15
	0	+	80	82		
	+	0	6	10		9
	+	+	78	82		

* The usual synthetic medium minus cysteine was used. + = ascorbic acid or cysteine • HCl added in amount indicated, 0 = not added. Solutions of compounds were sterilized by filtration and added to the medium after autoclaving and just before inoculation.

† Figures for growth represent turbidity. Lumetron readings of % light transmission with a 650 mμ filter were subtracted from 100. This was done for the purpose of showing increasing turbidities by higher figures. 0 = clear tube, 66 to 87 = heavy turbidity, near maximal or maximal growth. Incubation was at 37°C.

‡ Culture 1009 is included for comparison with the others. It failed to grow in the absence of cysteine whether or not ascorbic acid was supplied. It is representative of the 25 strains of lactobacilli which required cysteine whether or not ascorbic acid was present.

Recently, in continuing our study of cysteine requirement it was found that on addition of ascorbic acid to the chemically defined medium, cysteine was no longer required by certain lactobacilli. Others, though still requiring cysteine for growth, showed a more rapid growth rate in the presence of ascorbic acid. These findings are presented here.

Results. Table I gives the result of tests of cysteine requirement and of the substitu-

TABLE III. Effect of Different Amounts of Ascorbic Acid as Substitute for Cysteine in Growth of *L. fermenti*.

	L. fermenti strain numbers:													
	6					55			1002			1007		
	Growth at days													
Ascorbic acid,† mg/ml	2	3	4	7	10	2	3	7	2	3	7	3	4	5
0	0	0	0	0	0	0	0	0	0	0	0*	0	0	0
.01						0	0	0				0	0	0
.1	0	0	0	0	0	11	81		0	0	51	0	0	0
.3	0	0	0	0	75	63	85		6	59	67	0	19	64
.5									36	58	60	5	38	40
1.0	0	0	36	81		75	87		55	60	55	0	39	60
2.0	55	85				81	84		43	46	40	25	26	23

Turbidity is stated as in Table II.
* Occasional slow growth (40 to 50) appeared in from 7 to 10 days, but more often negative.
† Solutions were sterilized by filtration and added to the medium just before inoculation.

tion of ascorbic acid for cysteine as determined for our collection of lactobacilli. Of 36 lactobacilli which grew in a chemically defined medium containing both cysteine and methionine (but not ascorbic acid), only 6 were able to grow when cysteine was omitted; 30 failed to grow. The distribution of these cultures according to species, as determined in other work(1), is given in the table. In the last column it is seen that when ascorbic acid was supplied 11 cultures (the same 6 plus 5 more) were able to grow without cysteine. That is, in the presence of ascorbic acid a cysteine requirement was no longer exhibited by 5 strains: 1 *L. casei* and 4 *L. fermenti*.

Table II shows the individual responses to cysteine and ascorbic acid of 3 of the foregoing 5 lactobacilli and also, for comparison, of 1 other lactobacillus. All failed to grow when cysteine was omitted from the usual synthetic medium, but grew when 100 μg per ml of cysteine • HCl was supplied, though one of them (1007) always grew slowly. When ascorbic acid was supplied in place of cysteine growth of the first 3 organisms occurred. No. 1006 grew more slowly and not as fully as in the presence of cysteine, 55 responded to ascorbic acid as readily as to cysteine, and 1007 grew slowly when either cysteine or ascorbic acid, or both, was supplied. The needs of this strain for prompt growth are not known. The remaining 2 strains, not shown in Table II, grew promptly when sufficient ascorbic acid was supplied in place of cysteine. For all 5 strains there was an apparent substitution of

TABLE IV. Effect of Some Sulfur-Containing Compounds When Substituted for Cysteine.

S-containing compound	Amt, mg/ml	*L. casei* 1006	*L. fermenti* 55	*L. fermenti* 1007
0	0	2	0	0
Na thioglycolate	.1	75	85	69 (9)
	.3	74	74	73 (9)
	1.0	77 (3)	84 (6)	72 (7)
Mercaptosuccinic acid	.1	75	85 (3)	0
	.3	79	78	41 (6)
	1.0	79 (3)	77	50 (4)
2-Mercaptoethanol	.1	46	0	0
	.3	48	0	0
	1.0	52 (6)	0	0
Na thiosulfate pentahydrate	.1	75	83	0
	.3	74	84	0
	1.0	74 (3)	87 (7)	0

Results are for 48 hr at 37°C unless otherwise noted. Slower growth, when it appeared, is indicated by the day in parenthesis following the turbidity figure.
All tubes were held for 10 to 14 days before discarding.

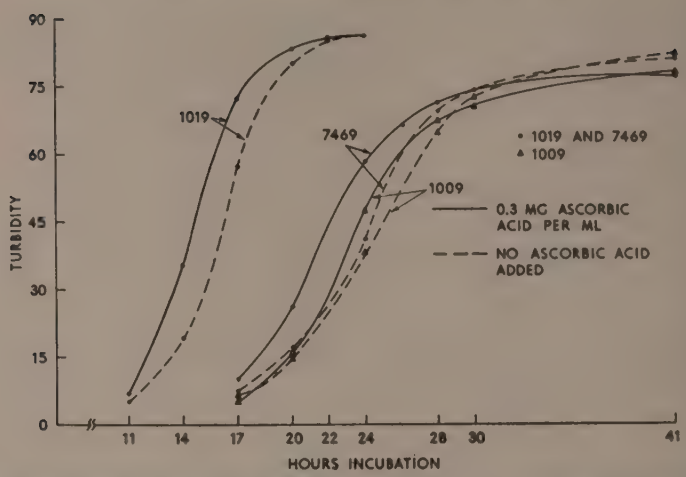

FIG. 1. Growth response of 3 lactobacilli to cysteine and to cysteine plus ascorbic acid. Cysteine • HCl, 100 μg/ml, was added to previously autoclaved medium. The cultures are *L. plantarum* 1019, *L. casei* ATCC 7469, and lactose negative *L. casei* 1009; 1019 and 1009 are of oral origin. These cultures all require cysteine irrespective of presence of ascorbic acid.

ascorbic acid for cysteine, though only partial in one case. These results were verified by repeated tests.

The effect of different amounts of ascorbic acid is shown in Table III. One strain regularly required as much as 2.0 mg per ml of medium for prompt growth while 2 others grew readily in the presence of 0.3 to 0.5 mg per ml. In certain instances there was evidence of some inhibition at the 2.0 mg level.

Other reducing substances were tested as substitutes for cysteine. In a medium lacking both cysteine and ascorbic acid, the cultures responded to additions of sodium thioglycolate or to mercaptosuccinic acid (Table IV). 2-Mercaptoethanol supported moderate growth of only 1 of 3, and sodium thiosulfate supported good growth of 2 of 3 cultures. Evidently ascorbic acid *per se* is not needed and the growth promoting effect can be secured by appropriate amounts of certain other reducing agents, though not all of the compounds are effective for all cultures. The apparent requirement for cysteine on the part of these lactobacilli is evidently a need for a reducing agent and not for cysteine, or ascorbic acid, as a structural entity.

Lactobacilli which require cysteine in the presence of ascorbic acid. Of the 36 lactobacilli studied, there remained 25 cultures which continued to exhibit a cysteine requirement in the presence of ascorbic acid, in contrast to the above cultures. For these 25 cultures cysteine apparently is needed for purposes other than the reducing effect. To determine what effect, if any, ascorbic acid might exert on growth of these lactobacilli in the presence of cysteine, observations of growth were made at different intervals with ascorbic acid added both to optimum and suboptimum amounts of cysteine. Addition of ascorbic acid to a medium containing sufficient cysteine for maximal growth produced a somewhat accelerated rate of growth, but not heavier final growth (Fig. 1). Similar results were secured with all other cultures tested in this manner: 1 *L. plantarum*, 4 *L. casei* of oral origin, and 1 *L. fermenti* which required cysteine in the presence of ascorbic acid.

For experiments with suboptimal amounts of cysteine 4 strains of lactobacilli were tested in the presence of 0, 1, 4, and 10 μg of cysteine • HCl per ml of medium. The last amount is sufficient for maximal growth of some lactobacilli but not enough for others. One set of tubes received 0.3 mg ascorbic acid per ml, the other received no ascorbic acid. It was found that the addition of ascorbic acid

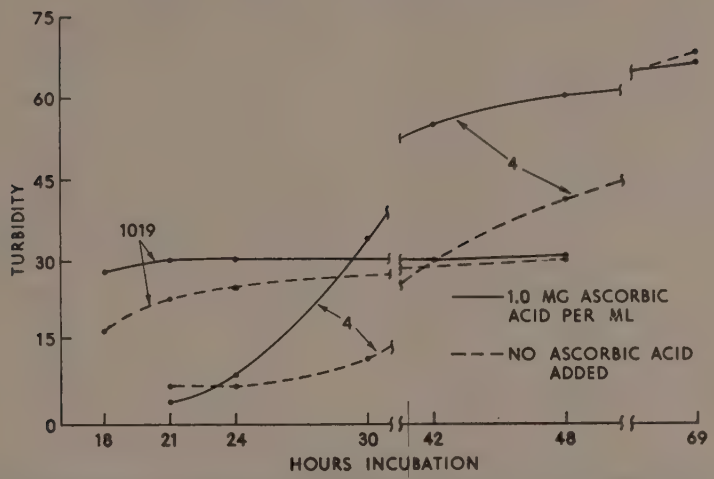

FIG. 2. Growth response of 2 lactobacilli to smaller amounts of cysteine • HCl, with and without ascorbic acid. Strain 4, a representative of lactose-negative *L. casei*, was grown in the presence of 4 μg cysteine • HCl/ml. Growth of *L. plantarum* is distinctly limited by the small amount of cysteine • HCl, 1 μg/ml, but in the presence of ascorbic acid growth is distinctly greater at the earlier data points.

accelerated the rate of growth in most instances, but there was little difference in the final amount of growth if the tubes were incubated a sufficient length of time (Fig. 2). Often several days were required to demonstrate this because growth was somewhat slower in the presence of the smaller amounts of cysteine • HCl.

Discussion. Four of the 5 cultures which do not need cysteine in the presence of appropriate reducing agents belong to the heterofermentative *L. fermenti* species. This seems significant from the standpoint of characteristics of this organism and its distinction from homofermentative lactobacilli. It suggests that a cysteine requirement is actually not as common among *L. fermenti* strains as former results had indicated and that *L. fermenti* differs from most homofermentative lactobacilli in this respect. In our collection of cultures, 6 of a total of 8 *L. fermenti* strains grew without cysteine. Two of the 6 (including the well-known ATCC 9338 strain) were able to grow in the usual chemically defined experimental media without additional reducing agents, but the other 4 grew without cysteine only in the presence of certain reducing agents. The remaining 2 strains required cysteine under all conditions tested. Whether other species of heterofermentative lactobacilli might exhibit deportment similar to the majority of *L. fermenti* cultures is not known since a sufficient number of strains has not been examined.

The ability of *L. fermenti* to dispense with preformed cysteine is in contrast to homofermentative lactobacilli, most of which exhibit a definite requirement for cysteine. In our collection of cultures only *L. plantarum* was somewhat of an exception to this trend in that 3 of 7 strains did not need cysteine. This difference in amino acid requirement is of interest in connection with another such difference recently noted. In previous work(1) it was found that threonine was either a definite requirement or stimulated growth of 5 *L. fermenti* strains, but was neither essential nor notably stimulatory for homofermentative lactobacilli: *L. casei,* lactose-negative *L. casei* and *L. plantarum* (including cultures designated *L. arabinosus* and *L. pentosus*). The present results indicate that the unessentiality of cysteine for most *L. fermenti* cultures under appropriate reducing conditions may be considered as another characteristic of this species, thus bringing to light one more

distinction between *L. fermenti* and homofermentative lactobacilli with respect to amino acid needs. A summary of other differentiating metabolic and cultural characteristics and their significance with respect to classification of the genus *Lactobacillus* has been given by Rogosa *et al.*(3).

The results have a possible bearing, too, on assay of cystine by microbiological methods. Not only is the rate of growth of many different lactobacilli accelerated by ascorbic acid in the presence of both suboptimum and optimum amounts of cysteine, but an apparent cysteine requirement of some cultures, mostly *L. fermenti,* disappears in the presence of ascorbic acid or certain other reducing agents.

Summary. 1. The requirement of some lactobacilli for cysteine, as determined in growth tests with usual chemically defined media, disappears when ascorbic acid in appropriate amounts, or other reducing substance, is incorporated in the medium. Of 5 strains of lactobacilli in this category encountered in a collection of 36 cultures, one was *L. casei,* the other 4 were heterofermentative *L. fermenti.* For these organisms the apparent cysteine requirement appears to be a need for a reducing substance and not for cysteine *per se.* Many *L. fermenti* strains evidently do not require cysteine though this may not always be apparent with some of the commonly-used synthetic media. 2. In contrast to *L. fermenti,* other lactobacilli—most *L. casei,* lactose-negative *L. casei, L. acidophilus,* and the majority of *L. plantarum*—require cysteine for growth in the presence of ascorbic acid. The rate of growth of these lactobacilli often is accelerated by ascorbic acid in the presence of either suboptimal or optimal amounts of cysteine. 3. Some implications of these findings concerning microbiological assay of cysteine and the distinguishing characteristics of heterofermentative *L. fermenti* and homofermentative lactobacilli are discussed.

1. Koser, S. A., and Thomas, J. L., *J. Infect. Dis.,* 1955, v98, in press.

2. Dunn, M. S., Shankmann, S., Camien, M. N., and Block, H., *J. Biol. Chem.,* 1947, v168, 1.

3. Rogosa, M., Wiseman, R. F., Mitchell, J. A., Disraely, M. N., and Beaman, A. J., *J. Bact.,* 1953, v65, 681.

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Comparative Study of Orosomucoid Preparations from Sera of Six Species of Mammals.* (22064)

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The presence of an electrophoretically and ultracentrifugally homogeneous acidic protein of very high carbohydrate content has been demonstrated in normal human plasma(1-4). This glycoprotein, designated orosomucoid (5,6), is one of the major components(5) of the "mucoprotein fraction" of human serum (6,7). "Mucoprotein" fractions have been demonstrated in the serum of many animal species(8-16). It appeared of some interest to determine whether proteins with characteristics of orosomucoid are also present in the blood of other species. In the present studies orosomucoid has been prepared from normal, pooled beef, guinea pig, horse, rabbit, and rat serum, and has been compared with that isolated from human plasma.

Materials and methods. Serum orosomucoid was separated by a procedure previously reported(1). To 500 ml of pooled serum from normal animals was added 500 ml of 0.1 M sodium acetate and 360 g of ammonium sulfate.† The precipitated proteins were

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† Erroneously reported previously(1) as 2.73 Molar.

removed by filtration after 16 hours at 4°C. The pH was reduced first to 4.9 and then to 3.7, the precipitated proteins again being removed by filtration after 16 hours at 4°C following each adjustment. Precipitation of the orosomucoid preparations was achieved by saturation of the filtrate with ammonium sulfate, the precipitate then being dialyzed and lyophilized. Since the animal sera were usually hemolyzed, it was necessary to refractionate 1% solutions of the orosomucoid preparations at least once to obtain a white product. Due to the loss of material during refractionation, an accurate estimate could not be made of the amount of orosomucoid in the sera of the different species. The isolated proteins were readily soluble in distilled water, in solutions of neutral salts, and in dilute or concentrated solutions of acids and alkalis. Acidified solutions were not coagulated by boiling.

Methods. The hexose content of the preparations was determined by the orcinol method of Sörenson and Haugaard(17), using a 1:1 galactose-mannose standard. Hexosamine was determined by the Rimington(13) modification of the Elson-Morgan method. "Sialic acid" was determined by the direct Ehrlich reaction as described by Werner and Odin(18), using "sialic acid" isolated from submaxillary mucin as a standard.‡ Nitrogen was determined by digestion and direct nesslerization(19). Protein was determined by the biuret method of Mehl(20), employing bovine serum albumin as a standard. All values are expressed on a moisture-free basis and were obtained by drying each preparation

TABLE I. Electrophoretic Mobilities of Orosomucoid at pH 8.6 and 4.5.

Species	Mobility × 10^{-5} cm²/volt/sec pH 8.5	pH 4.5
Human	−7.2*	−4.7*
Beef	−6.2*	−3.2*
Rabbit	−5.2†	−2.4†
Horse	−5.4†	−1.9†
Guinea pig	−5.8*	− .8*
Rat	−6.2*	− .8*

* Ascending boundaries with Tiselius apparatus.
† " " " Antweiler "

‡ We wish to thank Doctors I. Werner, L. Odin and G. Blix for samples of "sialic acid."

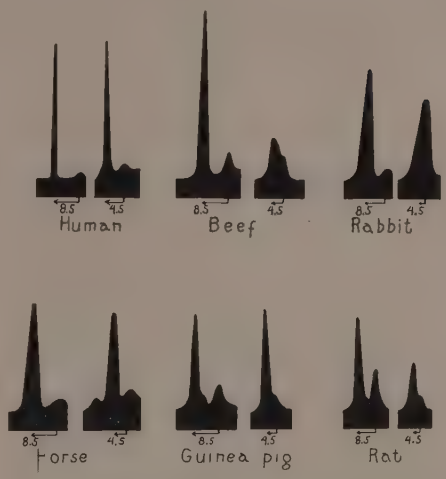

FIG. 1. Electrophoresis of orosomucoid preparations at pH 8.5 and 4.5. Ascending boundaries. Preparations from human, beef, guinea pig and rat with modified Tiselius apparatus. Rabbit and horse preparations with Antweiler micro-electrophoresis apparatus.

to constant weight at 105°C. Electrophoresis was carried out with a modified Tiselius apparatus, or with an Antweiler micro-electrophoresis apparatus using a veronal-sodium chloride buffer at pH 8.5, and an acetate-sodium chloride buffer at pH 4.5, ionic strength 0.1, as previously described(1).

Results. The results of the electrophoretic studies are shown in Table I and Fig. 1. Although electrophoretic homogeneity cannot be claimed for any of the preparations from animal sera, it is clear from the results at pH 4.5 that each preparation had an acidic protein as the major component.

Marked differences were apparent in the electrophoretic mobilities of the major component of different species, these differences being especially pronounced at pH 4.5. The fraction from human plasma had the greatest negative mobility at this pH (−4.7 x 10^{-5} cm²/v/sec), while the proteins from rat and from guinea pig serum had the lowest mobility at pH 4.5 (−0.8 x 10^{-5} cm²/v/sec). The major component of all preparations, however, was negatively charged at pH 4.5 and resembled, in this respect, the orosomucoid isolated from human plasma.

The results of the chemical analyses are

TABLE II. Partial Chemical Composition of Orosomucoid Preparations.*

Species	Hexose	Hexosamine	"Sialic acid"	Nitrogen	Protein
	%				
Human	16.5	12.0	11.2	10.1	63.0
Beef	13.9	7.9	10.9	11.9	77.0
Rabbit	12.3	9.1	6.9	12.0	72.0
Horse	12.3	7.0	6.8	12.5	73.0
Guinea pig	11.1	6.6	6.2	13.2	84.0
Rat	9.9	6.4	5.1	13.6	88.0

* Moisture-free basis.

summarized in Table II. The orosomucoid preparations of each species were characterized by high hexose, hexosamine, and "sialic acid," and by low nitrogen and protein contents relative to the major components of serum.

The current study has demonstrated that the blood of several species of animals contains a glycoprotein analogous to the orosomucoid of human plasma. The most pronounced features of this similarity are the high hexose, hemosamine, and "sialic acid" contents; the stability to heat; and the acidic nature of all the preparations. While the similarities in the fractions from various species are marked, it is apparent that there are notable differences among them. The carbohydrate contents, the ratios of hexose to hexosamine or "sialic acid" and of protein to carbohydrate show considerable variation from species to species. Although the electrophoretic data are far too limited to permit definite conclusions, it seems probable that there are significant species differences in the isoelectric points of orosomucoid, the I.E.P. of the rat and the guinea pig preparations apparently being higher than that of human orosomucoid.

Summary. Highly soluble, acidic, carbohydrate-rich proteins have been isolated from the serum of 6 animal species by a fractionation procedure employing ammonium sulfate. All of the preparations contained large amounts of hexose, hexosamine, and "sialic acid," and were not coagulated by heat. The major component of each preparation had an isoelectric point below pH 4.5, and resembled the orosomucoid isolated from human serum. Differences in chemical composition and in electrophoretic mobility were observed in preparations from different species.

1. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.*, 1950, v185, 561.

2. Smith, E. M., Brown, D. M., Weimer, H. E., and Winzler, R. J., *ibid.*, 1950, v185, 569.

3. Schmid, K., *J. Am. Chem. Soc.*, 1950, v72, 2816.

4. ———, *ibid.*, 1953, v75, 60.

5. Winzler, R. J., and Weimer, H. E., submitted for publication.

6. Winzler, R. J., *Methods of Biochemical Analysis*, 1955, v2, 279.

7. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.

8. Meyer, K., *Z. Physiol. Chem.*, 1942, v275, 16.

9. Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.*, 1944, v4, 417.

10. Catchpole, H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 221.

11. Pirani, C. V., and Catchpole, H., *Arch. Path.*, 1951, v51, 597.

12. Engel, M. B., *ibid.*, 1952, v53, 339.

13. Rimington, C., *Biochem. J.*, 1940, v34, 931.

14. Hewitt, L. F., *ibid.*, 1937, v31, 1534.

15. Hammerstrom, R. N., Adams, F. H., Bussman, J., and Lillehei, C. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 527.

16. Weimer, H. E., Carpenter, C. M., Redlich-Mochin, J., Little, M. S., and Nelson, E. L., *Physiol. Zool.*, 1954, v27, 341.

17. Sörensen, M., and Haugaard, G., *Biochem. J.*, 1933, v260, 247.

18. Werner, I., and Odin, L., *Acta Soc. Med. Upsaliensis*, 1952, v57, 230.

19. Toal, J. N., and Daniel, E. P., *J. Lab. and Clin. Med.*, 1950, v36, 950.

20. Mehl, J. W., *J. Biol. Chem.*, 1945, v157, 173.

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Enzymatic Porphyrin Synthesis in Harderian Glands and Other Organs and Tissues of Mice.* (22065)

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The fact that the Harderian gland of mice of certain strains(1-3), rats(4), and hamsters (5) contain high concentrations of porphyrin is well established. Some investigators assumed that the red fluorescent gland was a secretory or porphyrin-synthesizing structure (6,1,4). On the basis of injection experiments(3,5), one of us stated that the Harderian gland appears to be an excretory structure and that its red fluorescence thus reflects the level of porphyrin metabolism of other tissues. The object of the experiments reported in this paper was to compare the porphyrin-synthesizing potentialities of the Harderian gland with those of other tissues and organs. It was hoped that this would definitely determine whether the Harderian gland is a secretory or an excretory structure.

The testing of the porphyrin-synthesizing potentialities of Harderian gland was made possible by the recent discoveries that certain cellular enzymes have the ability to synthesize porphobilinogen, uro, copro, and proto porphyrins from δ aminolevulinic acid(7,8). In these experiments, measured amounts of aqueous extracts of tissue homogenates were tested to determine their content of porphyrin synthesizing enzymes. It was found that the Harderian gland of mice contained a high concentration of porphyrin-producing enzyme as compared with other tissues, and it thus appears to be a porphyrin-synthesizing organ.

Materials and methods. The tissues to be tested were taken from 12 male mice of the Strong A strain. The mice were raised and housed in glass cages containing wooden shavings. They were fed Purina Laboratory Chow and given water without restrictions. The mice were anesthetized with ether and the tissues dissected out in a cold room (6°C). The Harderian glands were removed as rapidly as possible with care being taken to exclude extraneous tissues. One hundred milligrams of Harderian gland tissue was weighed out and placed in a mortar with a small amount of coarse grinding sand. To this was added 2 cc of 50:50 buffer-saline solution. The mixture was homogenized, rinsed from the mortar with 3 cc of the buffer-saline solution, and centrifuged at 2500 r.p.m. for 15 minutes. The supernatant material was used as the enzyme solution. The same procedure was followed to prepare enzyme solutions of brain, liver, and the femur with its attached muscles. To each of the 24 Klett tubes (6 for each tissue to be tested) 1.4 cc of 0.01 M δ aminolevulinic acid buffer-saline solution was added to act as the substrate. The buffer-saline solution consisted of equal volumes of isotonic sodium chloride solution and buffer solution - pH 7.2. The amounts of enzyme solution from each tissue to be tested were pipetted into the 6 tubes as follows: Tube No. 1-none (control); Tube No. 2 - 0.1 cc; Tube No. 3 - 0.2 cc; Tube No. 4 - 0.4 cc; Tube No. 5 - 0.8 cc and Tube No. 6 - 1.6 cc. Buffered-saline was added to each tube to bring the volume to 3 cc. The tubes were then stoppered with cellophane-covered corks and incubated at 37°C for 24 hours. At the end of this period, 6.5 cc of concentrated HCl was added to each tube to stop the enzymatic reaction and to clear the solution of any precipitate. This produced a 25% HCl solution. The tubes were then allowed to stand overnight in a cold room (6°C). A solution of hematoporphyrin hydrochloride in 25% HCl was prepared as a standard to be used in a Klett-Summerson filter photometer to determine the amounts of porphyrin formed. These experiments were repeated 4 times.

Results. The variation in the amount of porphyrin produced by different tissue enzyme preparations is shown in Table I. From this table, it may be seen that the amount

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TABLE I. Porphyrin Production by Aqueous Extracts of Harderian Gland and Other Mouse Tissue Homogenates. 12 mice in each series.

	μ of porphyrin/10 cc of reaction mixture/24 hr*				
	Enzyme concentration				
Tissues	.1 cc	.2 cc	.4 cc	.8 cc	1.6 cc
Harderian gland	7.1 ± .6	12.8 ± .4	32.3 ± .6	49.5 ± .9	100.7 ± 1.5
Liver	5.5 ± .3	9.5 ± .9	28.5 ± 1.6	34.60 ± .6	73.4 ± 1.8
Muscle and bone	1.8 ± .01	3.4 ± .2	6.7 ± .3	8.7 ± .2	16.1 ± .4
Brain	.8 ± .01	2.5 ± .2	4.4 ± .3	5.2 ± .4	9.7 ± .4

* Mean ± S.E.

of porphyrin produced in the tube containing the extract of the Harderian gland homogenate was much greater than in any other tissue tested. This was first noted when the tubes with the various enzyme concentrations were examined in ultraviolet light.

Other tissues tested (liver, muscle-bone, and brain) also showed a definite ability to produce porphyrin. The amount of porphyrin produced by the enzyme preparation from these tissues was not as high as that found for the Harderian gland. The porphyrin-producing potentialities of liver tissue was less than that of Harderian gland but 5 and 7 times as great as muscle-bone and brain respectively.

Discussion. The fact that the Harderian gland homogenate enzyme preparation produces a greater amount of porphyrin than the other tissues examined indicates that the Harderian gland is a porphyrin-secreting structure; at least the enzymes for manufacture of porphyrin from simple compounds are abundant in it. Porphyrinogenic enzymes are even more concentrated in the Harderian gland than they are in the liver or kidney. Gibson, Neuberger, and Scott(9) had reported that the liver and kidney of rabbits and the erythrocytes of birds contained considerable quantities of δ aminoleuvulinic acid dehydrogenase. Since the liver is one of the primary organs involved in porphyrin and heme degradation, the presence of large amounts of porphyrin-synthesizing enzymes in this organ seems paradoxical.

The much lower quantities of porphyrin-producing enzyme in the muscle-bone marrow homogenates and in the brain may be related to the low percentage of cellular material involved in the homogenate. Also, our results may have been influenced to a slight degree by the presence of variable amounts of blood in the tissues, as no attempt was made to wash the blood out before the tissues were homogenized. However, this could not explain the great variation which we observed. Analysis of these results showed, as might be expected, that the differences observed are highly significant ($P<0.01$).

Summary and conclusions. 1. Buffered saline extracts of equal weights of mouse Harderian gland, liver, muscle-bone, and brain homogenates were prepared. 2. Various amounts of these extracts were tested for their capacity to produce porphyrin by adding 1.4 cc of a 0.01 M solution of δ aminolevulinic acid and incubating the mixture in a stoppered Klett tube at 37°C for 24 hours. 3. The Harderian gland displayed the greatest porphyrin-producing enzyme activity, whereas the liver was less than that of Harderian gland but 5 and 7 times as great as muscle-bone and brain respectively. 4. These results led to the conclusion that the Harderian gland is a porphyrin-secreting structure and probably not an excretory type of gland.

1. Derrien, E., and Turchini, J., *Compt. rend Soc. de biol.*, 1924, v91, 637.

2. Figge, Frank H. J., Strong, L. C., Strong, L. C., Jr., and Shanbrom, A., *Cancer Res.*, 1942, v2, 335.

3. Figge, Frank H. J., *ibid.*, 1944, v4, 465.

4. Thomas, J., *Bull. Soc. Chim. biol.*, 1938, v20, 1058.

5. Figge, Frank H. J., *A.A.A.S. Research Conference on Cancer*, 1945, 117-128 (Forest Ray Moulton, editor).

6. Grafflin, A. L., *Anat. Rec.*, 1941, v79, 25.

7. Dresel, E. J. B., and Falk, J. E., *Nature*, 1953, v172, 1185.

8. Granick, S., and Vander Schrieck, H. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 270.

9. Gibson, K. D., Neuberger, A., and Scott, J. J., *Biochem. J.*, 1954, v58, XLI.

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Effect of Heparin on Triglyceride and Free Fatty Acid Concentrations in Lymph.* (22066)

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The discovery by Hahn(1) that intravenously injected heparin abolished alimentary lipemia in dogs, is now well known. Clearing of lipemic serum did not occur *in vitro* upon addition of heparin itself(2,3); however, clearing of lipemic serum could be brought about by incubation with post-heparin plasma. Gofman *et al.*(4) in this laboratory have shown that plasma lipids are closely associated with a "spectrum" of lipoprotein molecules, which are distinguishable in the ultracentrifuge by virtue of their different densities. The effect of heparin *in vivo* on these molecules was found to be a conversion of the species of high flotation rates to those of lower flotation rates(5). Shore, Nichols and Freeman(6,7) in this laboratory also showed that clearing of a lipemic substrate by post-heparin plasma *in vitro* was accompanied by the release of free fatty acids. Robinson and French also reported the release of fatty acid when lymph was incubated with post-heparin plasma(8). Grossman *et al.*(9) have subsequently found that free fatty acid concentration in the plasma was increased after the injection of heparin, especially in the presence of lipemia. Similar observations have been made in both animals and humans in this laboratory.

It appears from the foregoing that the free fatty acids produced during *in vitro* or *in vivo* clearing of lipemia are at least partly the result of glyceryl ester hydrolysis, activated in some way by heparin. It is of interest to know whether such glyceryl ester hydrolysis can be demonstrated in the lymphatic system as well as in the blood. In the present communication evidence for lipolysis of triglycerides with release of fatty acids in the lymphatic system is presented.

A. *Materials.* 1. Animals: Male New Zealand white rabbits of body weight between 3 and 3½ kg and male Long Evans rats, 250-350 g were used. All animals were fasted for 12 to 18 hours before cannulation, unless otherwise stated. 2. Anesthetics: Nembutal, 50 to 60 mg/kg body weight administered intravenously for rabbits; 30 to 40 mg/kg for rats intraperitoneally. Ether was used to supplement nembutal anesthesia. 3. Heparin, aqueous solution, 10000 units/ml was used intravenously in doses of 15 mg/kg for rabbits and 5 mg/kg for rats. 4. C. P. ether, methanol, chloroform and carbon disulfide were used (J. T. Baker Chem. Co.). Commercial grade hexane was redistilled. 5. Trielaidin was prepared in this laboratory by reaction of elaidoyl chloride and glycerol. Infrared spectrum of partially purified product used for feeding indicated that small amounts of partial glycerides and free fatty acid were present. These do not interfere with the experiment, since presumably they would be formed in the digestive process.

B. *Methods.* 1. Collection of lymph: a) Cannulation of rat lymphatics—method of collection is essentially the same as that employed by Bollman *et al.*(10). b) Cannulation of rabbit lymphatics: In different experiments lymph was collected from 3 different regions, thoracic duct, hepatic lymph vessel and intestinal lymphatic vessel. A section of polyethylene tubing was used as cannula. For collection of thoracic duct lymph, tubing was cannulated into thoracic duct at junction of left subclavian and left internal jugular veins. For collection of hepatic lymph tubing was inserted into hepatic lymphatic duct at point approximately 1 cm caudal to liver near hepatic artery. Intestinal lymph was collected from main intestinal lymphatic vessel, along coeliac artery, just above left adrenal gland. Cannulae were left in place post-operatively and lymph in each case was collected continuously 48 hours after rabbits had recovered from anesthesia. During each study lymph

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TABLE I. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Thoracic Lymph of 5 Rabbits.

Time after heparin (hr)	Triglycerides (mg/100 ml)						Free fatty acids (mg/100 ml)					
	1	2	3	4	5	Mean	1	2	3	4	5	Mean
−2.0	102	—	104	—	—	103	11	—	10	—	—	11
.0	100	106	101	114	113	106	13	14	12	10	8	12
.5	58	59	81	88	72	72	52	61	31	36	21	38
1.0	36	45	32	42	32	37	21	29	29	30	38	29
2.0	28	33	21	32	40	31	18	21	20	20	18	19
2.5	40	39	25	22	55	36	16	20	20	19	21	20
3.5	50	—	—	25	78	51	20	—	—	19	26	22
4.5	—	93	—	—	—	93	—	18	—	—	—	18
6.0	—	—	94	—	—	94	—	—	17	—	—	17
12.0	—	—	—	103	—	103	—	—	—	18	—	18
24.0	—	100	—	—	—	100	—	16	—	—	—	16

samples were collected at 30 minute intervals. 2. Lipids of lymph were extracted and separated by method essentially similar to that of Borgstrom(11,12). Extracted lipids were separated into 3 fractions by elution from silicic acid columns. Triglycerides and free fatty acids were determined quantitatively by infrared spectrophotometry(7). For determination of elaidic acid, free fatty acids were separated from glycerides by extraction from petroleum ether solution with dilute alkali in 50% methanol(11). Content of elaidic acid was measured in both glycerides and free fatty acids by infrared spectrophotometric measurements of absorption band at 10.3 μ. Basis of this method has been given by Shreve *et al.*(13). Since amounts of material and percentages of elaidic acid were both small in our experiments, we used a base line method of calculation instead of equations developed by those authors. Results by the two procedures agree well when total sample is large enough. For smaller samples, in particular free fatty acids of pre-heparin lymph, only maximum values have been quoted. As a result of fatty acid exchange in processes of digestion and absorption, glycerides subsequently found in lymph are presumed to contain partially elaidinated fat molecules. The analysis gives equivalent amount of trielaidin.

Results. 1. Thoracic Duct Lymph (Rabbit and Rat). All lymph samples collected from the thoracic duct of rabbits before administration of heparin, 15 mg/kg intravenously, were visibly turbid. Post-heparin samples were clear. Table I summarizes changes in triglycerides and free fatty acids in thoracic duct lymph of 5 rabbits. Concentration of triglyceride decreased about 35% within first half hour after administration of heparin. The decrease reached its maximum, an average of 74%, in 2 hours. After that time its concentration increased gradually to the original level. Free fatty acids, on the other hand, increased by about 360% in the first 30 minutes. The increase in free fatty acids after administration of heparin does not compensate for the decrease in triglycerides. The conditions of the *in vivo* experiment are not such that an accurate balance of triglyceride and fatty acid changes can be expected, and also incomplete hydrolysis of triglyceride probably occurred. Similar results obtained in rats are shown in Table II. Smaller doses of heparin were effective in this species, about 5 mg/kg being comparable with 15 mg/kg in the rabbit.

TABLE II. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Thoracic Lymph of 5 Rats.

Time after heparin (hr)	Triglycerides (mg/100 ml)						Free fatty acids (mg/100 ml)					
	1	2	3	4	5	Mean	1	2	3	4	5	Mean
0	207	218	214	220	239	220	25	39	28	35	48	35
1	116	112	160	128	221	147	140	90	73	57	160	104
2	105	125	135	105	224	138	160	192	56	101	213	144
3	—	—	—	—	127	127	—	—	—	—	128	128

TABLE III. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Intestinal Lymph of Rabbits.

Time after heparin (hr)	Triglycerides (mg/100 ml) 1	2	3	Mean	Free fatty acids (mg/100 ml) 1	2	3	Mean
0	316	289	308	304	19	8	17	15
1	287	290	292	289	24	10	16	17
2	150	200	187	179	25	18	18	21
3	61	148	123	111	16	19	30	22
4	34	41	80	52	19	23	30	23

The average maximal drop (47%) in triglyceride concentration was at 3 hours post-heparin. Maximal increase in free fatty acid concentration (290%) was observed in 2 hours.

2. Intestinal lymph (Rabbit). The fat content of intestinal lymph was much higher than that of thoracic and hepatic lymph. The results of heparin injections are presented in Table III. It is notable that elevation of free fatty acid concentration is much less than for thoracic duct lymph. Also the lowering of triglycerides proceeds to a greater extent and for a longer period of time. 3. Hepatic lymph of normal rabbits was watery clear. The fat content of hepatic lymph was lower than that of lymph collected from either thoracic duct or intestinal lymph. Sixty minutes after administration of heparin, there was a slight change in triglycerides and a relatively large percentage increase in free fatty acids. Table IV shows triglyceride and free fatty acid contents of hepatic lymph before and after administration of heparin.

4. Trielaidin Feeding. To learn more about the origin of free fatty acids produced in lymph after heparin administration, tracer experiments were performed using trielaidin as a labeled fat. Rats cannulated in the thoracic duct as described were fed (by stomach tube) a suspension of 50-100 mg trielaidin in 2 ml of non-fat milk. Heparin was given 3-3½ hours after this feeding, and lymph samples were collected and analyzed. Results are summarized in Table V.

TABLE IV. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Hepatic Lymph of Rabbits.

Time after heparin (hr)	Triglycerides (mg/100 ml) 1	2	3	Mean	Free fatty acids (mg/100 ml) 1	2	3	Mean
0	65	70	60	65	7	10	7	8
1	65	45	25	45	70	31	32	44
2	45	33	20	33	76	54	46	58
3	29	—	16	23	40	—	43	42
4	51	—	18	35	39	—	40	39
5	56	—	—	56	41	—	—	41

5. *In vitro* effects. If lipolysis occurs in post-heparin lymph *in vivo* it may be inferred that the active factor is present in such lymph and should be demonstrable by *in vitro* test. Such confirmation is provided by two observations. (1) Pre- and post-heparin rabbit hepatic lymph were incubated with a fatty substrate prepared from rat lymph by centrifuging 3½ hours at 20000 r.p.m. In each case 0.2 ml of top 1 ml from this centrifugation was mixed with 1 ml of lymph to be tested for lipolytic activity and 0.5 ml of saline, and the mixture incubated 3 hours at 38°C. Glycerides and free fatty acids were determined in the same way as described for the *in vivo* experiments. (2) Analyses were made of fresh pre- and post-heparin rabbit thoracic duct lymph and compared with results obtained on same samples after they remained at 4°C for 3 weeks. The results for these two experiments are presented in Table VI.

Discussion. The data that have been presented demonstrate an effect in the lymph of both rabbits and rats, following intravenous injection of heparin, that is evidently parallel to the effect in blood. The triglyceride content drops, and the amount of free fatty acids increases. It is further indicated that in the absence of excess fat influx from the intestine (*i.e.*, in fasted animals) a predominant share of free fatty acids found in thoracic duct lymph has arrived there via the hepatic lymph channel. This suggests an implication of the liver as principal source of fatty acids under these conditions. However, if trielaidin is fed and heparin is given during absorptive state, 10-20% of free fatty acids formed consist of elaidic acid. Thus it appears that hydrolysis of some newly absorbed fat has taken place. This hypothesis is further supported by ability of post-heparin lymph to produce lipolysis *in vitro*. However, an alternative explanation which has not been ruled out is that absorp-

TABLE V. Changes in Elaidin and Free Elaidic Acid in Rat Lymph after Heparin. (Concentrations are in mg/100 ml.)

Exp.	Time with respect to heparin inj.	Total glyceride	Elaidin (equivalent trielaidin)	Total free fatty acids	Free elaidic acid
1	−2	299	26	—	—
	0	540	75	51	<5
	1	314	40	192	12
2	−3	236	51	37	<5
	0	304	62	61	6
	2	137	40	189	20
3	−3	189	32	57	<8
	0	207	47	29	<8
	2	116	35	156	16
4	−3½	264	59	30	<8
	0	570	175	50	<8
	2	365	143	136	26

tion process from the intestine may have been so altered that a larger fraction of fatty acids is passed into lymph without being reconstituted as glycerides.

Summary. The glyceride and free fatty acid contents of thoracic, intestinal, and hepatic lymph of rabbits, and thoracic lymph of rats were determined before and after intravenous administration of heparin. The principal effects observed were the following: 1. The turbidity that was always observed initially in thoracic or intestinal lymph was diminished or disappeared. Hepatic lymph was not originally turbid. 2. In all cases the glyceride content was reduced and free fatty acids increased. 3. Relative amount of glycerides was highest in intestinal lymph, and the change in that component was markedly greater than in hepatic lymph. 4. Hepatic lymph had a low initial content of glycerides, and showed a relatively larger change in free fatty acids. 5. Trielaidin was fed to rats and heparin given intravenously during absorptive phase. Elaidic acid was detected in free fatty acids subsequently appearing in lymph. Post-heparin rabbit lymph was capable of producing lipolysis of a fatty substrate *in vitro*.

TABLE VI. Lipolytic Activity of Post-Heparin Lymph *In Vitro*. (Concentrations are in mg/100 ml.)

	Glycerides		Free fatty acids	
	Initial conc.	Final conc.	Initial conc.	Final conc.
Exp. 1				
Pre-heparin	260	258	39	40
Post-heparin	233	196	75	115
Exp. 2				
Pre-heparin	116	112	10	14
Post-heparin	75	43	25	57

Exp. 1. Incubation of rat lymph lipids with rabbit hepatic lymph for 3 hr at 38°C.

Exp. 2. Rabbit thoracic duct lymph kept at 4°C for 3 wk.

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1. Hahn, P. F., *Science*, 1943, v98, 19.

2. Weld, C. B., *Canad. M. A. J.*, 1944, v51, 578.

3. ———, *ibid.*, 1946, v54, 71.

4. Gofman, J. W., Lindgren, F. T., Elliott, H. A., Mantz, W., Hewitt, J., Strisower, B., Herring, V., and Lyon, T. P., *Science*, 1950, v111, 166.

5. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S., *Circulation*, 1951, v4, 666.

6. Nichols, A. V., Freeman, N. K., Shore, B., and Rubin, L., *ibid.*, 1952, v6, 457.

7. Shore, B., Nichols, A. V., and Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

8. Robinsin, D. S., and French, J. E., *Q. J. Exp. Physiol.*, 1953, v38, 233.

9. Grossman, M. I., Palm, I., Becker, G. H., and Moeller, H. G., *U. S. Army Med. Nutrition Lab. Rep.*, 1954, No. 131.

10. Bollman, J. L., Cain, J. C., and Grindlay, J. H., *Lab. and Clin. Med.*, 1948, v33, 1349.

11. Borgstrom, B., *Acta Physiol. Scand.*, 1952, v25, 101.

12. ———, *ibid.*, 1952, v25, 111.

13. Shreve, O. D., Heether, M. R., Knight, H. B., and Swern, Daniel, *Analytical Chem.*, 1950, v22, 1261.

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Daily Variations in Mitotic Activity of Adrenal Cortex, Thyroid and Oral Epithelium of the Rat.* (22067)

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(Introduced by Franz Halberg.)

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Daily physiologic periodicity seems to characterize mitotic activity in the oral epithelium(1,2) as well as in a variety of other tissues from plants and animals (for review see 3 & 4). These daily changes in mitotic activity may be influenced by daily periodicity in various environmental factors which affect the organism. But intrinsic, partly hormonal mechanisms also may contribute to the 24-hour spacing of peaks in mitotic activity(2,4). In this latter connection, the following questions were explored in this study. First, whether day-night differences may be detected in the mitotic activity of certain endocrines under the standardized environmental circumstances and at those times of day at which significant day-night differences have been demonstrated in the oral epithelium(2). If day-night differences existed in the mitotic activity of the glands studied, it also was of interest to know whether the times of "high" and "low" in number of mitoses were roughly the same in endocrine and non-endocrine tissues.

Materials and methods. For 3 weeks prior to their sacrifice, 32 Sherman albino rats weighing about 135 g were kept in single cages in a room illuminated from 6:00 to 18:00, darkened from 18:00 to 6:00, and maintained at 24 ± 1°C and 49 ± 2% relative humidity. A commercial diet (Lab Blocks, Allied Mills, Chicago) and tap water were available to the rats *ad libitum* since weaning and throughout the period of standardization. A subgroup composed of 16 rats was decapitated between 7:00 and 7:30 ("Day-rats") and another subgroup of 16 rats between 21:30 and 22:00 ("Night-rats"). The adrenal glands, the thyroid and part of the oral epithelium were immediately fixed by means of Zenker-Formol. Serial sections, 4 μ thick, were then prepared and stained with Hematoxylin-Eosin. Mitotic activity in every section from each tissue and rat was then evaluated. In the case of the adrenal cortex, mitotic activity was related to area-units of 10 mm^2. For the thyroid, mitoses were counted in 150 oil-immersion fields(83 ± 1 cells per field). In the oral epithelium, mitoses were counted in units of 10 mm length of basal membrane. The length of basal membrane was measured by the use of a special device which will be described elsewhere(5).

Results. A summary of results in Fig. 1 shows the considerable variability of the data for individual rats, which are given by the height of the columns graphed. More important, some considerable day-night differences in mitotic activity also become apparent from a comparison of the horizontal lines, which denote the means in mitotic activity by day and by night. For the thyroid and for the oral epithelium, there is a day "high" and a night "low" in number of mitoses. By contrast, a night "high" and a day "low" appear to characterize mitotic activity in the adrenal cortex of the nocturnally active rat.

Discussion. These data seem to demonstrate, first, a 24-hour periodicity of mitosis in the thyroid and adrenal of the rat. Second, the data also reveal that daily mitotic periodicity in the thyroid is synchronized with the 24-hour spacing of mitosis in the oral epithelium. Mitotic periodicity in the oral epithelium, in turn, has been shown to be synchronized with 24-hour periodicity of mitosis in other tissues(2) and with the 24-hour rhythms in eosinophil count and in rectal temperature (1,2). It also has been shown that the adrenals are essential for the maintenance of 24-hour rhythms in eosinophil count(4,6) and in some aspects of hepatic phospholipid metabolism(7). If, as seems likely(4), the

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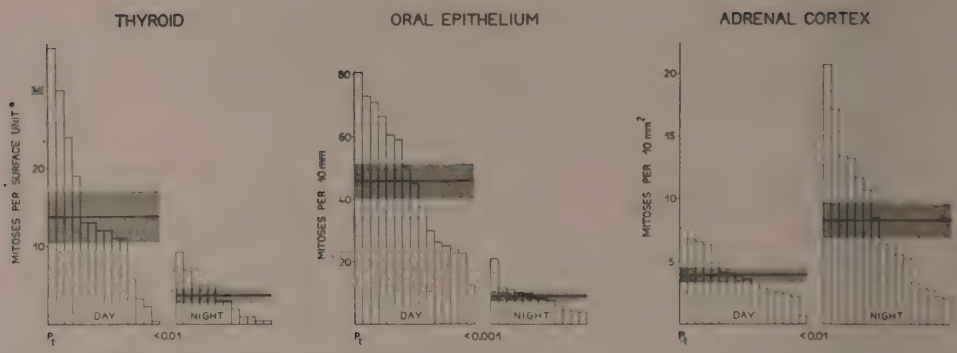

* Mitoses/150 oil-immersion fields.

FIG. 1. Mitotic activity at two times of day in tissues investigated. (Columns show data for individual rats; horizontal lines and shaded areas denote means ± 1 S.E.; day = 7:00-7:30; night = 21:00-21:30.)

adrenal constitutes a fundamental mechanism of physiologic daily periodicity, the phase-difference noted herein between its mitotic periodicity and the 24-hour spacing of other rhythms may call for a detailed analysis of adrenal mitotic activity (with more frequent sampling during the 24-hour period).

Summary. Daily periodicity characterizes mitotic activity in the adrenal, the thyroid and the oral epithelium of male Sherman albino rats, kept under standardized environmental circumstances. The daily times of "high" and "low" in number of mitoses are roughly synchronized for the thyroid and the oral epithelium, while the 24-hour periodicity in mitotic activity of the adrenal cortex shows a different timing.

1. Mühlemann, H. R., Zander, H. A., and Halberg, F., *J. Dent. Res.*, 1954, v33, 459.

2. Halberg, F., Zander, H. R., Houglum, M. W., and Mühlemann, H. R., *Am. J. Physiol.*, 1954, v177, 361.

3. Bullough, W. S., *Biol. Revs. Cambridge Phil. Soc.*, 1952, v27, 133.

4. Halberg, F., *Journal Lancet*, 1953, v73, 20.

5. Marthaler, T. M., *Oral Med. and Oral Path.*, in press.

6. Kaine, H. D., Seltzer, H. S., and Conn, J. W., *J. Lab. and Clin. Med.*, 1955, v45, 247.

7. Halberg, F., Barnum, C. P., and Vermund, H., *J. Clin. Endocrinol. and Metab.*, 1953, v13, 871.

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Oxytocic Action of Rat Hypothalamus Extracts. (22068)

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Vogt(1) confirmed the presence of vasopressor, antidiuretic and oxytocic activities in extracts of dog hypothalamus. According to her results the hypothalamus differs from the posterior pituitary in that the relative oxytocic activity of the hypothalamus is much lower than the expected value, the P/O ratio (pressor activity/oxytocic activity) being 14.6. Inasmuch as Lawler and du Vigneaud (2) have shown that oxytocin-free vasopressin possesses an oxytocic activity corresponding to 5% of the pressor activity, Vogt(1) suggests that the oxytocic substance extracted from the hypothalamus may be pure vasopressin. This interpretation is of importance in view of the findings of Bargmann and Scharrer(3) on production of hormones of the pars nervosa of the hypophysis by the neuro-

secretory nuclei of the hypothalamus. These observations led Vogt(1) to deduce that either the oxytocin is chemically changed by the hypothalamus or this organ produces oxytocin and its nuclei the vasopressin.

Methods. The comparative effect of several crystalline proteolytic enzymes: pepsin, chymotrypsin, trypsin and carboxypeptidase, on extracts of hypothalamus and posterior pituitary of the same rat was studied in order to determine whether the oxytocic activity of the hypothalamus might be due to a substance similar to either oxytocin or vasopressin. Croxatto(4) has shown that the oxytocic activity is destroyed by chymotrypsin, but it is not affected by carboxypeptidase, pepsin and trypsin; on the other hand vasopressin is inactivated by chymotrypsin and also by crystalline trypsin. Lawler and du Vigneaud (2) using a highly purified preparation of vasopressin have confirmed the latter finding. Extracts were obtained from the anterior hypothalamic region of male and female adult rats, comprising a triangular area based on the posterior boundary of the optic tractus, from which portions of the tuber cinereum were definitely excluded. The small portion tested contained the supraopticus and paraventricularis nuclei. The tissue thus obtained from 2 or 3 rats was ground with micronized quartz in a mortar, the temperature maintained at 0°C. The ground tissue was suspended in 0.9% NaCl, in the proportion of one ml per hypothalamus. The clear supernatant fluid obtained on centrifuging at low temperature was divided in 2 equal portions, to one of which 2 to 3 mg of enzyme were added. Both samples were incubated at 37°C for one to 6 hours at pH 7.3, except that in the case of pepsin the pH was 2.5. After incubation the extract was neutralized and tested on rat uterus. Some of the incubation mixtures were brought to pH 2.5 and deproteinized by addition of 10 volumes of alcohol, centrifuged, the alcohol in the supernatant evaporated and the residue taken up in Ringer's solution. The posterior lobes of the hypophysis of the same animals were subjected to the same treatment as the hypothalamus.

TABLE I. Oxytocic Activity (mU) Anterior Hypothalamus Extracts.

Before incubation	After incubation: No enzyme	After incubation: Enzyme added		Hr
.75	.5	.7	Pepsin	1
	.5	.5		1
	.5	.75		5
1.-	1.0	1.0		5
.5	.5	.5		5
	1.5	.5	Chymotrypsin	1
	*1.5	1.5		1
	1.0	.5		1
	1.0	.1		2
	1.0	.1		2
.5	.4	0		6
	.2	0		6
	.5	.75	Trypsin	4
.5	.2	.2		6
	.2	.2		6
	.40	.5		6
	.2	.4		6
.5	.3	.3		6
.6	.5	.5	Carboxypept.	2
	.3	.3		4
	† .2	.4		4
	.3	.3		6

Extracts were incubated at pH 7.3 except in the case of pepsin the pH was 2.5; in (*) the pH was 3 and in (†) the pH was 8.2.

Results. The effect of pepsin, carboxypeptidase, trypsin and chymotrypsin on extracts of hypothalamus was the same as that observed for extracts of the posterior pituitary (Table I). Chymotrypsin was the only enzyme that caused a complete or almost complete loss of the oxytocic activity of hypothalamus and posterior pituitary extracts. No decrease in oxytocic activity of both extracts was produced by the other enzymes tested. In some experiments trypsin, carboxypeptidase and pepsin produced a slight increase of the oxytocic activity of both extracts. Incubation of the extracts at pH 7.3 with no addition of enzyme resulted in an appreciable loss of oxytocic activity by both extracts.

If one takes into consideration the specificity of the enzymes tested, our results would strongly support the hypothesis of the chemical identity of the substances from the hypothalamus and the posterior pituitary of rats. Contrary to the assumption of Vogt(1) regarding dog hypothalamus, the oxytocic activity of rat hypothalamus appears to correspond to that of a substance like oxytocin rather than one like vasopressin.

Summary. A study of the effect of pepsin, carboxypeptidase, trypsin and chymotrypsin on the oxytocic activity of extracts of the rat hypothalamus and posterior lobe of the hypophysis. Both extracts show similar behavior. Chymotrypsin is the only enzyme that produces complete or almost complete loss of the oxytocic activity. No decrease in oxytocic activity is produced by the other enzymes. Incubation of the extracts at pH 7.3 without enzymes results in an appreciable loss of oxytocic activity by both extracts.

1. Vogt, M., *Brit. J. Pharmacol.*, 1953, v8, 193.
2. Lawler, H., and du Vigneaud, V., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 114.
3. Bargmann, W., and Scharrer, E., *Amer. Scientist*, 1951, v39, 255.
4. Croxatto, H., *Rev. Med. y Aliment.*, 1942, v5, 259.

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Antithyroid Properties of 2-Amino, 5-Nitrothiazole in Rats.* (22069)

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The report that 2-amino, 5-nitrothiazole caused the complete suppression of sexual manifestations and functions in fowl(1-3) has been explained as being the result of inhibition of secretion of pituitary gonadotropin(3). Although the effect of the compound appeared to be restricted to fowl(3), interest in a possible antigonadotropic substance that is not related to the sex-hormones has prompted the present investigation of 2-amino, 5-nitrothiazole utilizing the rat as the experimental animal.

Materials and methods. Eighteen litters of Long-Evans female rats from 45 to 49 days of age were assigned to 4 groups so that each group contained 18 rats from different litters and so that each group had the same average body weight. At this time 2 groups were ovariectomized and 2 groups were sham-operated. One operated group and one sham-operated group was started the next day on commercial 2-amino, 5-nitrothiazole† added to the diet at a concentration of 0.3%. All other rats received the same diet (commercial laboratory chow‡ that is adequate in iodine) with no drug added. All rats were maintained on their respective diets and tap water *ad libitum* until sacrifice 30 days later. The intact animals were given a 2 μc tracer dose of carrier free I^{131}§ by the intraperitoneal route and sacrificed 24 hours later. The operated animals were handled exactly as above except that they were sacrificed 48 hours post I^{131} administration. At sacrifice the body, pituitary, thyroid, adrenal and uterus weights were recorded and the thyroid glands were placed in 2 cc of Bouin's fluid and assayed for radioactivity in a well-type scintillation counter against 3 aliquots of the original I^{131} as prepared for injection. The radioactivity measurements were expressed as the percentage of injected dose found in the sample and the counting statistics were such that the resulting probable error was less than 1%. The thyroid glands were then sectioned and stained for histological study. In another experiment 30 day-old male Sprague-Dawley rats were assigned to 3 groups of 15 animals so that the average body weight of all groups was the same. These rats were maintained

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† "Enheptin", a commercial product containing aminonitrothiazole not less than 20%, soybean oil meal, not more than 80%, crude protein not less than 33%, crude fat not less than 0.3% and crude fiber not more than 7%, sold by American Cyanamid Co. (Lederle Laboratories Division), New York City.

‡ Purina Laboratory Chow. Ralston Purina Co., St. Louis, Mo.

§ Obtained from Oak Ridge National Laboratory.

TABLE I. Effect of Feeding 2-Amino, 5-Nitrothiazole (0.3% of Diet Adequate in Iodine for 30 Days) to Intact and Ovariectomized Female Long-Evans Rats on the Body Weight, Adrenal Weight, Thyroid Weight, Pituitary Weight, Uterus Weight and Percent of Administered I^{131} Accumulated by the Thyroid Gland (24 Hr between Isotope Administration for Intact Animals and 48 Hr for Ovariectomized Animals). Mean ± stand. error reported.

Treatment	No. of animals	Body wt (g)	Adrenal (mg)	Thyroid (mg)	Pituitary (mg)	Uterus (mg)	I^{131} (%)
Intact	18	154 ± 4	40.3 ± 2.2	9.2 ± .5	6.20 ± .24	181 ±23	6.35 ± .48
Intact + 2-amino, 5-nitrothiazole	18	142* ± 4	31.8† ± 1.7	11.1† ± .4	5.96 ± .24	141 ±17	3.12† ± .27
Ovariectomized	17	161 ± 4	38.6 ± 2.0	9.6 ± .5	7.35 ± .20	40 ± 3	4.39 ± .30
Ovariectomized + 2-amino, 5-nitrothiazole	16	144* ± 4	31.3† ± 1.3	11.4† ± .5	6.84 ± .26	35 ± 2	.60† ± .11

* P_{05} when tested by t-test against mean immediately above.
† P_{01} " " " " " " " .

TABLE II. Effect of Feeding 2-Amino, 5-Nitrothiazole (0.30%) or Purified 2-Amino, 5-Nitrothiazole (0.06%) in a Diet Low in Iodine to Male Sprague-Dawley Rats for 8 Days on Body Weight, Testis Weight, Thyroid Weight, Pituitary Weight, and Percent of Administered I^{131} Accumulated by the Thyroid Gland (18 Hr between Isotope Administration and Sacrifice). 15 animals in each series.

Treatment	Body wt (g)	Testis (g)	Thyroid (mg)	Pituitary (mg)	I^{131} (%)
.06% 2-amino, 5-nitrothiazole	91 ± 1	1.21 ± .04	7.5 ± .3	3.86 ± .12	17.1 ± 1.6
Low iodine	92 ± 2	1.22 ± .32	6.6* ± .2	3.84 ± .22	25.4† ± 1.6
.30% 2-amino, 5-nitrothiazole	92 ± 2	1.16 ± .18	8.1† ± .4	3.82 ± .10	16.7† ± .9

* † Same as in Table I.

on a diet low in iodine‖ for 8 days(4). One group received commercial 2-amino, 5-nitrothiazole added to the diet at a concentration of 0.30%, another group received the purified compound¶ at a concentration of 0.06%, while one group received no drug treatment. All animals were maintained on these diets and tap water *ad libitum* until sacrifice. Eighteen hours before sacrifice each rat received a tracer of I^{131} and radioactivity of the thyroid gland determined as before.

Results. The feeding of a commercial preparation of 2-amino, 5-nitrothiazole (0.30% of a diet adequate in iodine) for 30 days to intact or ovariectomized Long-Evans rats had no effect on pituitary, or uterus weights. The 2-amino, 5-nitrothiazole treated rats had smaller body and adrenal weights, larger thyroid weights, and lower 24 hour and 48 hour thyroidal accumulation I^{131} values than non-treated rats (Table I).

Male Sprague-Dawley rats fed either a commercial preparation of 2-amino, 5-nitrothiazole or a purified preparation of the compound (0.30% or 0.06% respectively of a diet poor in iodine) for 8 days had no effect on body, testis or pituitary weights. However, the 2-amino, 5-nitrothiazole treated rats had larger thyroid glands and a lower 18-hour thyroidal accumulation of I^{131} than the non-treated rats (Table II).

In all groups treated with 2-amino, 5-nitrothiazole the thyroid glands gave a histological picture of columnar follicular cells and less densely staining colloid that is consistent with effects given by treatment with antithyroid compounds of the thiouracil type (Fig. 1, 2).

Discussion. 2-amino, 5-nitrothiazole had no effect on gonad function, as judged by testis weights or uterus weight, when administered to male or female rats. This is in agreement with previous reports(3) since the effectiveness of 2-amino, 5-nitrothiazole in suppressing gonad function was limited to fowl and could not be extended to mammals.

‖ Remington Diet No. 348. Each 100 g contains: wheat gluten, 16 g; dried yeast, 4 g; corn meal 78 g; $CaCO_3$, 1 g; and NaCl, 1 g.

¶ 2-amino, 5-nitrothiazole, Lot No. 251T2F, assay 97.2% pure, contributed by Lederle Laboratories.

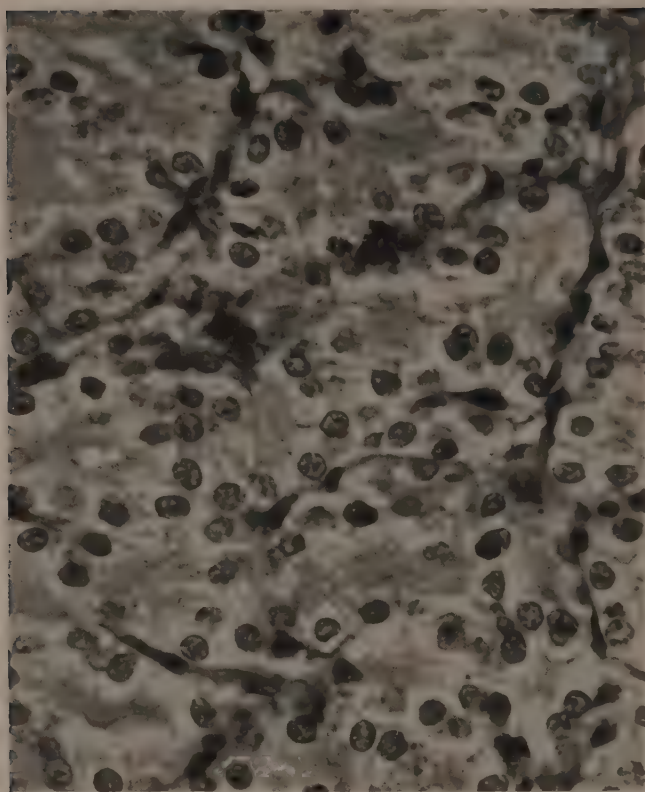

FIG. 1. Thyroid gland from female Long-Evans rat that had been fed 0.30% 2-amino, 5-nitrothiazole for 30 days. Bouin fixation, hematoxylin and eosin. (×600.)

However, there seems to be little doubt that 2-amino, 5-nitrothiazole had a definite antithyroid action in these rats since the thyroid weight and follicular cell height was increased and the thyroidal accumulation of I^{131} was lowered in rats treated with the compound. It is not surprising that 2-amino, 5-nitrothiazole possesses antithyroid properties since it is well known, in man at least, that aminothiazoles possess definite antithyroid properties that produce an increase in thyroid weight and a lowering of I^{131} uptake. In fact, the aminothiazoles have been used for the control of hyperthyroidism(5). It seems likely that the other changes noted in the aminonitrothiazole-treated rats, *i.e.*, a depressed body and adrenal weight, might also be ascribed to an induced hypothyroidism since these changes are not inconsistent with changes produced by hypothyroidism(6). Indeed, it is possible that the suppression of sexual function in fowl noted following treatment with the compound might also, at least in part, be ascribed to a relative lack of thyroid hormone since various degrees of hypothyroidism may contribute to a lack or regression of gonad function and the anterior pituitary responses to changes in gonad function(7). The fact that the gonadal suppression noted in 2-amino, 5-nitrothiazole treated fowl has not been attributed to a decreased thyroid function does not detract from the foregoing suggestion because the only tests reported on thyroid function in fowl treated with 2-amino, 5-nitrothiazole were tests designed to show if the compound suppresses the pituitary production of thyrotropin and were not designed to test the possibility of a direct action of 2-

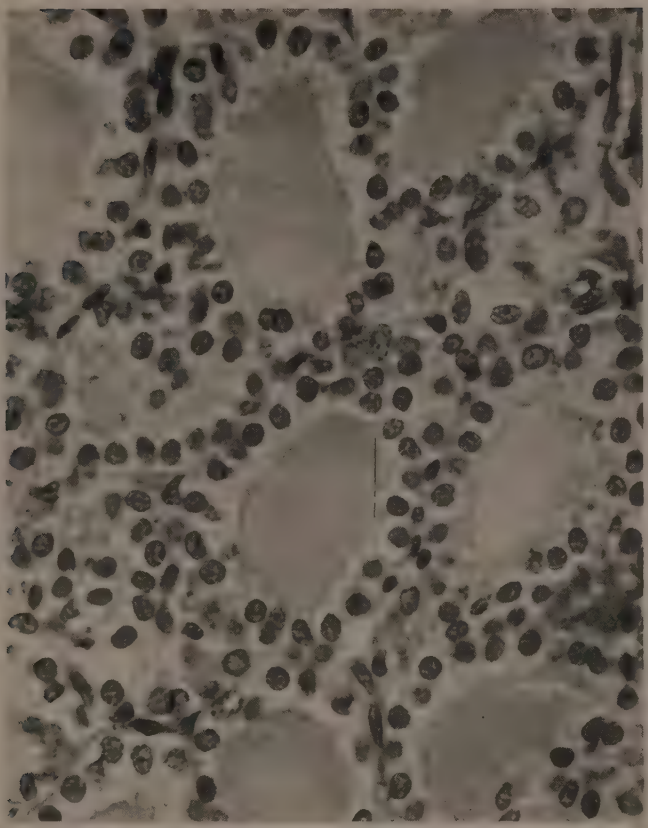

FIG. 2. Thyroid gland from control female Long-Evans rat of same age as in Fig. 1. Bouin fixation, hematoxylin and eosin. (×600.)

amino, 5-nitrothiazole on the thyroid gland or its production of thyroid hormone. Also, the reports that this compound interrupts egg laying(8,9) are consistent with the suggestion that 2-amino, 5-nitrothiazole might produce abnormalities in pituitary-thyroid function by reason of its antithyroid properties.

Summary. Rats fed 2-amino, 5-nitrothiazole had larger thyroid glands and a lower thyroidal accumulation of I^{131} than non-treated rats. It is suggested that the antithyroid properties of this compound might explain the inhibition of secretion of pituitary gonadotropin that has been reported following the administration of 2-amino, 5-nitrothiazole to fowl.

1. Hudson, C. B., and Pino, J. A., *Poul. Sci.*, 1952, v31, 1017.

2. Pino, J. A., and Hudson, C. B., *ibid.*, 1953, v32, 650.

3. Pino, J. A., Rosenblatt, L. S., and Hudson, C. B., PROC. SOC. EXP. BIOL. AND MED., 1954, v73, 311.

4. Remington, R. E., *J. Nutrition*, 1937, v13, 223.

5. Perrault, M., and Bovet, D., *Ann. d'endocrinol.*, 1954, v6, 86.

6. Money, W. L., 1954, Brookhaven Symposia in Biology No. 7, Office of Technical Services, Department of Commerce, Washington 25, D.C.

7. McGavack, T. H., 1951, *The Thyroid*, C. V. Mosby Co., St. Louis, Mo.

8. Grumbles, L. C., Boney, W. A., and Turk, R. D., *Am. J. Vet. Res.*, 1952, v13, 1952.

9. Pino, J. A., *Poul. Sci.*, 1955, v34, 540.

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Leukopenic and Thrombocytopenic Effect of Hypothermia in Dogs. (22070)

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(Introduced by G. M. Guest.)
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Serious cardiac arrhythmias and cessation of respiratory center activity are two major complications of hypothermia which have caught the attention of investigators studying the effects of cooling. The purpose of this report is to describe another complication observed in a high proportion of our experiments. Leukopenia and a thrombocytopenia often developed in dogs which were subjected to rapid cooling; this marked reduction in the number of thrombocytes may be related to the hemorrhagic phenomena which were also encountered in many of the animals. During early investigations of hypothermia in this laboratory we noted that a large proportion of the dogs bled from the gastro-intestinal tract during the first 48 hours after rewarming(1). This loss of blood was a complication seen more frequently in the dogs subjected to experiments in hypothermia than in a similar group of animals subjected to major operative procedures with normal body temperature. In the course of another group of hypothermia experiments a second manifestation of this hemorrhagic diathesis was noted. Dogs that were cooled to the range of 18° to 26°C were subjected to exploratory thoracotomy. In most of these animals the surfaces of the wounds oozed blood in a way not observed in the course of operations upon animals with unimpaired coagulation mechanisms. This faulty coagulation was no doubt the cause of the unusually high incidence of hemothorax in the animals which survived the experiment. Several earlier reports mentioned intestinal tract bleeding as a complication in dogs that had been cooled(2,3). While this complication was manifest in more than one-half of the animals cooled in our laboratory, the impression gained from publications is that others have not observed this type of bleeding so frequently. The mechanism of the diathesis has not been explained, although Ross has noted prolongation of the clotting time in his experiments with cooling by an extracorporeal technic(4).

There is also a clinical aspect to this problem. Even though it cannot be stated in absolute terms at this time, it appears that impairment of the coagulation mechanism may develop in patients subjected to hypothermia as currently employed for cardiovascular surgery(5). It seemed advisable, therefore, to plan a group of experiments in rapid cooling with special attention to the effects upon the blood cellular elements, as the first step in an attempt to elucidate the problem of post-hypothermia bleeding in dogs.

Method. The mongrel dogs used were 8 months old or older; those with infection or malnutrition were excluded from the group for these experiments. Excitation of the animals during the administration of the anesthetic was avoided as far as possible. Pentobarbital sodium was administered intravenously in average dose of 250 mg. The trachea was intubated at once. In the more active animals this was facilitated by the administration of small amounts of ether by open drop. Cyclopropane and oxygen were administered thereafter, and this proved to be an ideal anesthetic in all experiments in which hypothermia was induced. However, the animals with normal body temperature in the control series required additional doses of diphenhydramine hydrochloride (Demerol) to maintain anesthesia at a satisfactory plane. All of the dogs breathed actively during the period of observation and no mechanical ventilator was employed. Animals were supported in the prone position, tilted so that the extended hind legs were at a level 6 inches higher than the head. The dorsal surface of the thorax was shaved and cracked ice was

packed about all of the body except the head and neck. Brine was circulated through this loose ice pack to speed the cooling. Additional procedures in the experiments involved only minor surgery. A ligature was passed around the trachea and tied so that no gas escaped around the in-lying tracheal catheter. The venous pressure was measured through a catheter inserted into the superior vena cava. Samples of venous blood drawn after the animal was anesthetized were obtained through the same catheter; the blood was allowed to drip into heparin tubes and syringes were not employed. The blood pressure was measured with a mercury manometer, attached to a cannula in the femoral artery. Temperature changes were measured by a thermometer inserted into the rectum. Samples of venous blood were kept chilled in a pan of cracked ice. The counts of erythrocytes, platelets(6) and leukocytes(7), as well as the preparation of blood smears and determination of hematocrit(8), were completed within 4 hours after the samples were collected.

Results. Control experiments were carried out on 5 dogs. The standardized method for anesthesia was used for this group, but it did not prove as satisfactory as when it was employed in the hypothermia series (see Method). The conditions of the experiment did not vary in any other respect; the animals in the control group had their backs shaved and were subjected to the same procedures as those employed in the hypothermia group for cannulation of the trachea and an artery and vein. Observations were continued over a period slightly longer in the series of control animals than in the group of 19 that were placed in cracked ice. The average period of observation was 157 minutes for the control group and 100 minutes for the group subjected to cooling.

Acidosis which was most likely respiratory in origin, was of comparable severity in both groups of animals. This fact was ascertained by the measurements of pH and carbon dioxide content in samples of arterial blood drawn at regular intervals during the course of the experiments. The average of the lowest rectal temperatures observed in the animals in the chilled group was 24.3°C.

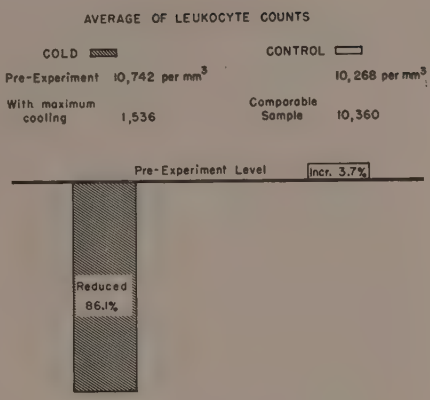

FIG. 1.

Changes in the hematocrit were similar to those that have been reported on many occasions by earlier investigators. The dogs that were cooled had some hemoconcentration with an average increase in hematocrit of 9 vol. %. The mean corpuscular hemoglobin concentration fell somewhat with the average change 1.7%. The mean corpuscular volume increased with the average rise 7.5(9) cubic micra.

There was a marked change in the total leukocyte count produced by hypothermia. Fig. 1 tabulates the averaged results in the cooled group for comparison with the averaged changes in the controls. An 86% reduction in leukocyte count was associated with the lowered body temperature. A differential count of cells was not made from every sample. However, the changes were consistent in all samples that were examined completely. The percentage of lymphocytes in the smears increased and the percentage of neutrophils fell to approximately one-third of the value obtained at normal temperature. The conditions of the control experiments resulted in a small increase (3.7%) in the leukocyte count. This change in the control series is not statistically significant.

The total eosinophil count was also altered in these experiments. In Fig. 2 the results in the hypothermic group are compared with the results from dogs at approximately normal

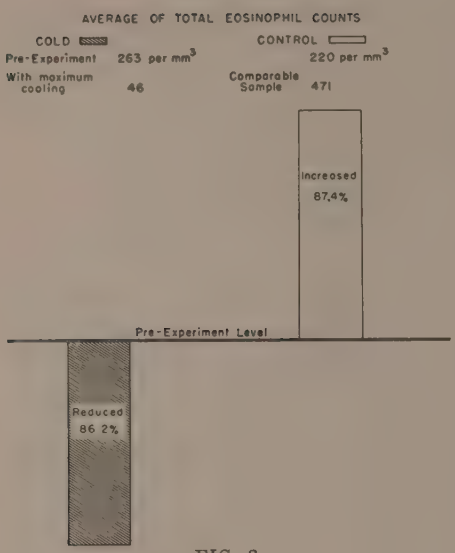

FIG. 2.

body temperature.* The average of the fall in total eosinophil count was 86.2% in the hypothermic group. In the controls, however, this count was increased 87.4%.

Another outstanding change observed in these experiments was in the platelet count (Fig. 3). The change observed in the control experiments was a reduction in the count which averaged 16.5%. The dogs that were cooled had an 81.7% reduction in the count

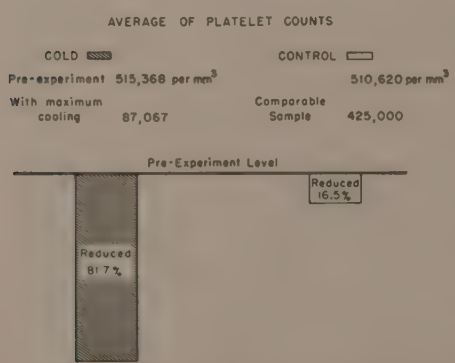

FIG. 3.

* The range of temperatures was from 36° to 37°C. Slight cooling resulted from washing, shaving, and the anesthetized state.

of platelets. In several of the experiments no platelets could be found in the samples of blood drawn at the time of the maximum cooling effect. The samples of blood remained uncoagulated when allowed to stand for two hours in Lee-White tubes at room temperature.

Summary. 1. Attention is drawn to the complication of impaired coagulation encountered in experimental hypothermia in dogs. 2. The method used for anesthetizing and rapidly reducing the body temperature by packing in cracked ice is described. 3. The following changes were associated with hypothermia: The leukocyte count was reduced an average of 86%, the total eosinophil count was reduced an average of 86%, and the platelet count was reduced an average of 81%. 4. The coagulation mechanism was not studied directly, but data from these experiments suggest that the strong thrombocytopenic effect observed may be a factor in the impaired coagulation.

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1. Selye, H., *Stress*, Acta, 1950, p. 688.

2. Parkins, W. M., Jensen, Jay M., and Vars, H. M., *Ann. Surg.*, 1954, v140, 3.

3. Deterling, Ralph A., Jr., Nelson, Eleanor, Bhonslay, Shivaji, and Howland, William, *A.M.A. Arch. Surg.*, 1955, v70, 1.

4. Ross, D. N., *Guy's Hosp. Rep.*, London, 1954, v103, 116.

5. Shumway, Norman E., and Gliedman, Marvin L., *Coronary Perfusion for Longer Periods of Cardiac Occlusion under Hypothermia.* Presented at 35th annual meeting, Am. Assn. for Thor. Surg., Atlantic City, April 24-26, 1955.

6. Dameshek, William, *Arch. Int. Med.*, 1932, v50, 579.

7. Pilot, Martin L., *Am. J. Clin. Path.*, 1950, v20, 870.

8. Guest, George M., Siler, Vinton E., *J. Lab. and Clin. Med.*, 1934, v19, 7.

9. Helmsworth, J. A., Stiles, Wm. J., and Elstun, Wesley, *Soc. for Vascular Surg.*, Atlantic City, June 5, 1955.

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In vitro Anaphylaxis in the Sensitized Mouse Uterus. (22071)

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Anaphylaxis in the mouse has been studied by many workers, and most of their findings indicate that histamine plays little or no role in anaphylactic shock in this species. Using the Schultz-Dale technic of *in vitro* anaphylaxis, this paper is concerned with an attempt to rule histamine in or out as being part of the basic mechanism in the reaction and to study the effect of pertussis vaccine on *in vitro* anaphylaxis.

Materials and methods. Schultz-Dale technic in mouse anaphylaxis. Fourteen virgin female Swiss mice over 3 months old were injected subcutaneously with 0.6 ml of alum precipitated egg albumin† prepared by the method of Proom(1). This amount contained 15 mg of the antigen. The mice were used 2 to 4 weeks later, at a time when, as judged by gaping of the vaginal orifice, they were in estrus, and the uterus consequently larger.‡ After sacrificing the animal by stretching its neck, the uterus was immediately removed and placed in a modified Ringer's solution(2). It was kept at 0°-4°C until used, usually within 2 hours. Two aerated tissue baths, each containing 45 ml of the Ringer's solution maintained at 38°-39°C were set up. One-half of one uterine horn was suspended in one bath; one-half of the other horn in the other bath.§ After 30 minutes, the lever on each of the strips was connected to a kymograph, and the pattern of normal contractions obtained. Crystalline egg albumin‖ was then added to each bath to a concentration of 15 μg per ml of bath. All additions were made with a 3-inch needle attached to a tuberculin syringe, with the tip of the needle bent in a manner to insure spraying of the liquid. After contraction of the strip, which invariably occurred within 30 seconds after the first addition of antigen, the tissues were washed, the normal contractions recorded, and a second addition of the same amount of egg albumin made to test for desensitization. When desensitization had been effected, to test the reactivity of the tissue, acetylcholine¶ was added to a concentration of 10 μg per ml, and the contraction recorded. At least one tissue strip from each of the 14 mice was found to react to egg albumin. In 13 of the mice, at least 2, and sometimes 3 or 4 reacted; in only one mouse was just one piece reactive. Duplicate contractions of tissues from the same uterus were usually but not always of a similar magnitude. The occasional dissimilarity in reactivity of different portions of the uterus is in line with similar observations on the guinea pig ileum(3). A typical contraction is shown in Fig. 1.

Tissues from 12 of the mice did not react on the second addition of antigen; but those from the other 2 mice were not desensitized until 3 additions of antigen had been made. Similar observations have been reported for the guinea pig(4). After desensitization, all of the tissues reacted to acetylcholine in a concentration of 10 μg per ml of bath. The uteri from 10 normal mice were similarly tested. In no case was there a reaction to egg albumin; but in every case a reaction occurred in response to the 10 μg per ml of acetylcholine.

Role of histamine and acetylcholine in mouse anaphylaxis. This experiment was de-

* This work supported by a Grant-in-Aid from the American Cancer Society upon recommendation of Committee on Growth of the National Research Council.

† Albumin, Egg Impalpable Powder, J. T. Baker Chem. Co.

‡ After this first experiment was completed, it was found that administration of an estrogen 4 to 5 days prior to testing resulted in uniformly large uteri. In all other experiments to be reported mice were injected intramuscularly with 8.8 γ of *Progynon-B* (Schering Corp.) in mineral oil prior to testing.

§ In some instances, the other 2 pieces were tested in a similar manner.

‖ Armour and Co. preparation.

¶ Acetylcholine chloride, Matheson Co.

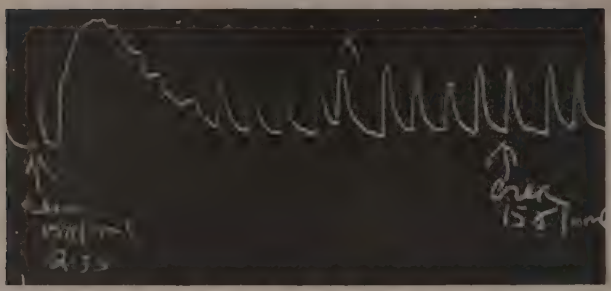

FIG. 1. Typical anaphylactic contraction of actively sensitized mouse uterus. Arrow = crystalline egg albumin added 15 μg/ml. × = contraction following wash.

signed to determine the role of histamine and acetylcholine by blocking reactions due to these drugs with Benadryl and atropine, respectively. It was first demonstrated that these drugs could block the reactions when histamine or acetylcholine were added to the sensitized mouse uterus; but that they could not block the reactions when antigen was added to the sensitized uterus. Ten mice actively sensitized as before and 3 mice passively sensitized by the intra-abdominal injection 4 hours prior to testing of 0.4 ml of pooled serum from actively sensitized mice were used in this study. This antiserum contained sufficient antibody for a positive precipitin reaction when antigen in a concentration of 0.01 mg per ml was added.

In preliminary studies it was found that histamine in a concentration of 50 μg of histamine** base per ml of bath was necessary to produce a uniform uterine contraction of the sensitized uterus. Benadryl†† in a concentration of 0.4 to 0.8 μg per ml of bath was always sufficient to prevent the reaction to histamine. Strips not responding to histamine in the presence of Benadryl would, after washing, respond to a corresponding dose of histamine.

It was also shown that atropine‡‡ in a concentration of 2 μg per ml of bath was invariably sufficient to prevent the reaction to 10 μg or more of acetylcholine per ml of bath.

** Crystalline histamine diphosphate, Eimer and Amend, dissolved in phosphate buffered saline, pH 7.2.

†† Parke, Davis and Co. preparation.

‡‡ Obtained from local pharmacy.

The technic of testing was as described previously. It was first established that a positive reaction to egg albumin could be obtained on one-half of one uterine horn. Then 2 fresh pieces of the same uterus were set up in the 2 water baths. To one of these Benadryl was added to a concentration of usually 0.4 μg per ml of bath; to the other, atropine was added to a concentration of 2 μg per ml of bath. After about one minute, the antigen was added as before. In some instances, the fourth piece of tissue was tested in the presence of both atropine and Benadryl.

FIG. 2. Persistence of anaphylactic contraction in presence of atropine and Benadryl. × = contraction following wash.

In all 13 mice—10 actively and 3 passively sensitized—neither Benadryl nor atropine inhibited or diminished the anaphylactic contraction (Fig. 2).

Uterine mast cells. In view of reports indicating that histamine may be released from tissue mast cells(5,6), an experiment was done to determine whether there were any changes in the uterine mast cells subsequent to *in vitro* anaphylaxis. Two strips of uterus from an actively sensitized mouse were placed in Ringer's solution as usual. After shocking a strip *in vitro,* it was placed in 10% formalin; the second, non-shocked strip served as a control. The tissues were prepared by a standard histological technic. In the 3 times the experiment was done, the mast cells were abundant and easily identified, but there were never any discernible changes in their morphology after shock.

Effect of pertussis vaccine on anaphylactic and histamine sensitivity in vitro. It has been shown that an injection of pertussis vaccine increases both histamine and anaphylactic sensitivity in the intact mouse. This experiment was done to determine whether similar findings could be observed when the tests were done *in vitro.* Five groups of female Swiss mice over 3 months old were used in this experiment. The first group of 18 were not injected, and served as controls. Mice in the other 4 groups were all injected subcutaneously with the 0.6 ml of alum precipitated egg white as before. One of these 4 groups received no further treatment. The other 3 groups received pertussis vaccine as follows: 9 mice were injected intra-abdominally with 0.1 ml of pertussis vaccine§§ immediately after the egg white injection; a group of 12 received 0.1 ml of pertussis vaccine 4 to 5 days prior to testing for sensitivity; and the last group of 8 received 0.2 ml of pertussis vaccine 4 to 5 days prior to testing. On the 10th, 11th, 12th, and 13th day following the egg white injection, proportionate numbers from each of the 5 groups were tested for anaphylactic sensitivity as previously described until all had been tested. They were tested for histamine sensitivity by recording the reaction to 50 μg of histamine base per ml of bath.

§§ Pertussis vaccine, Cutter lot No. E5452; 0.1 ml contains 6 x 10^9 organisms.

TABLE I. Effect of Pertussis Vaccine on Anaphylaxis and Histamine Sensitivity *in Vitro.**

Exp.	Normal	Ea	Ea + .1 pertussis 4-5 days earlier	Ea + .2 pertussis 4-5 days earlier	Ea and pertussis injected together
A +† H +		8/12‡	3/12	2/8	3/9
A + H −		1/12	2/12	2/8	0/9
A − H +		2/12	6/12	3/8	3/9
A − H −		1/12	1/12	1/8	3/9
Total H +	3/18	10/12	9/12	5/8	6/9
Total A +		8/12	5/12	4/8	3/9

* All mice received estrogen intramuscularly 4-5 days prior to testing.
† A = anaphylaxis; H = histamine—50 γ/ml of bath.
‡ No. positive/No. tested.

In these, and in all following experiments, only one strip was tested if the reaction were positive; 2 strips if negative. The results of these experiments are recorded in Table I.

The results of the experiment on the effect of pertussis vaccine on anaphylactic and on histamine sensitivity are expressed in Table I. To determine any difference in sensitivity to histamine and to anaphylaxis, the groups were compared statistically using the method of χ^2. Each group was compared with every other group in the 2 categories.

Analysis of the data reveals that pertussis vaccine had no effect on anaphylactic sensitivity as tested *in vitro* when given 4 days prior to testing at 2 dosage levels; or when administered with the sensitizing dose of antigen. Nor does pertussis vaccine have any more effect on histamine sensitivity as measured *in vitro* than does the injection of the sensitizing antigen alone. None of the treated groups were statistically different from each other, but all were statistically different from the normals.

Discussion. Numerous publications have questioned the role of histamine in mouse anaphylaxis. The relative insensitivity of the

intact mouse to histamine(7-9), the suggestion that the body of the mouse does not contain sufficient histamine to produce a reaction (7), the observation that antihistaminic drugs have little if any effect on anaphylaxis (10), and the inability to establish a correlation between histamine and anaphylactic sensitivity in this species(10,11), all indirectly suggest that histamine is not concerned in anaphylaxis in the mouse.

By using the Schultz-Dale technic of *in vitro* anaphylaxis, it has been shown in this study that the isolated tissue, like the intact animal, is very resistant to histamine; and that the sensitized mouse uterus reacts to the specific antigen to the same extent in the presence of a demonstrably anti-histaminic drug as it does in the absence of that drug. Furthermore, no change in the tissue mast cells, believed to be involved in the release of histamine(5,6), was manifested after the *in vitro* anaphylactic reaction. These findings strengthen the hypothesis that histamine is not concerned in mouse anaphylaxis.

The reaction also proceeds to its full extent in the presence of atropine, a finding which would argue against an important role for acetylcholine.

The fact that pertussis vaccine increases both histamine and anaphylactic sensitivity in the intact mouse has been amply confirmed (8,9,12). Studies on the isolated tissues, however, reveal that anaphylactic sensitivity to the egg white antigen was not increased; and that the increase in the histamine sensitivity was of the same order if the animal received the antigen alone, or the antigen plus pertussis organisms. In light of the finding that the tissues were all similarly sensitive, this suggests that the effect of pertussis vaccine in increasing anaphylactic sensitivity in the intact animal must be through alteration of some mechanism other than an immunological one. The finding that the egg white injection increases histamine sensitivity to the same extent that pertussis vaccine does (Table I) is difficult to explain. Many bacterial antigens have been tested(10,13) to determine whether or not they increased sensitivity to histamine, but only those prepared from *Brucella abortus* were found to have any effect. Parventjev(14), however, has shown that injection of a mouse with Sarcoma 180 tends to increase the histamine sensitivity.

Summary. 1. The Schultz-Dale technic for demonstrating anaphylaxis *in vitro* was used successfully on uteri from egg white sensitized mice. 2. The anaphylactic reaction persisted in the presence of either atropine or Benadryl, thus ruling out any major role for histamine or acetylcholine in mouse anaphylaxis. 3. The uterine mast cells were found to be morphologically unaffected by *in vitro* anaphylaxis. 4. Pertussis vaccine administered under varying conditions did not increase *in vitro* sensitivity to anaphylaxis. Immunization with egg white alone increased histamine sensitivity *in vitro* as much as did the pertussis vaccine.

We are indebted to D. L. Smith for advice on pharmacological procedures; and to C. D. Fink for statistical appraisal of the data.

1. Proom, H., *J. Path. Bact.*, 1943, v55, 419.

2. Burn, J. H., *Practical Pharmacology*, Blackwell Scientific Publications, Oxford, 1952, p15.

3. Kempf, A. H., and Feinberg, S. M., *J. Allergy*, 1948, v19, 247.

4. Kendall, A. I., and Shumate, F. O., *J. Inf. Dis.*, 1930, v47, 267.

5. Riley, J. F., *J. Path. Bact.*, 1953, v65, 471.

6. Fawcett, D. W., *J. Exp. Med.*, 1954, v100, 217.

7. Mayer, R. L., and Brousseau, D., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 187.

8. Parventjev, I. A., and Goodline, A., *J. Pharm. and Exp. Therap.*, 1948, v92, 411.

9. Pittman, M., *J. Inf. Dis.*, 1951, v89, 296.

10. Malkiel, S., and Hargis, B. J., *J. Allergy*, 1952, v23, 352.

11. Fink, M. A., and Rothlauf, M. V., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 336.

12. Malkiel, S., Hargis, B. J., and Feinberg, S. M., *J. Immunol.*, 1953, v71, 311.

13. Kind, L. S., *J. Immunol.*, 1953, v70, 411.

14. Parventjev, I. A., *Proc. Am. Assn. for Cancer Res.*, 1955, v2, 38 abstr.

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Precipitin Analysis of C-Reactive Protein by Gel Diffusion.* (22072)

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C-reactive protein (C-RP), an abnormal protein present in the "acute phase" serum, has been under investigation for the past several years. This substance, which forms a precipitate with the non-type specific C-polysaccharide of the pneumococcus in the presence of Ca^{++}(1), was found by electrophoresis, to migrate between the α_2 and β globulin fractions(2). The C-reactive protein was separated from the human serum(3) and was prepared in crystalline form from human serous fluids(2). A number of investigations have shown the presence of C-RP to be a very sensitive indicator of activity in rheumatic fever(4-6). Evidence has been presented that C-RP may be present in cancer(7) and in acute myocardial infarction(8). In rheumatoid arthritis and active tuberculosis, the C-RP is significantly correlated with seromucoid(9). Small amounts of this abnormal protein may be detected in human serum by a precipitin test utilizing specific antiserum from rabbits hyperimmunized with purified C-RP.

The present study was carried out during the course of studies on the biological properties of C-reactive proteins. With the use of agar gel diffusion technics it was possible to obtain quantitative and qualitative values of C-RP in the serum of seventy-one patients with a variety of illnesses.

Procedure. C-RP antiserum—Three New Zealand Red rabbits (2 kg) were injected intravenously, twice a week for 3 weeks, with 0.5 cc of a solution containing 1 mg/cc of crystalline C-RP. 1.5 cc of Freund's adjuvant(10) was incorporated with 1 mg of crystalline C-RP and injected subcutaneously into 2 separate abdominal sites at the time of the first intravenous injection. On the fourth week the rabbits were bled and the sera were preserved with merthiolate in a final concentration of 1:10,000. The *crystalline C-RP* was prepared from 2 liters of fluid obtained by thoracocentesis from a patient with Hodgkin's disease. The isolation and crystallization of C-RP was performed following the method described by Wood *et al.*(11). The total yield of crystallized material was 28 mg. *Human sera* were obtained from the serology laboratory at the Mount Sinai Hospital, Chicago, from 71 patients with a wide variety of illnesses. The age of the patients ranged from 12 to 74 years. *The quantitative precipitin technic* for the determination of antibodies in rabbit C-RP antisera was that described by Kabat and Mayer(12). The amount of antigen was added in slight excess to produce a total antibody precipitation and the total nitrogen precipitated was determined by the microkjeldahl technic. Each analysis was carried out in duplicate. Tests of the supernatant fluid showed that the dilution of crystalline C-RP reacted with antibodies in the region of antigen excess. The amount of antibody nitrogen precipitated at the equivalence zone from each rabbit antiserum was, respectively 0.652 mg, 0.615 mg, and 0.485 mg per cc of antiserum. *Agar precipitin (Oudin) technic*—The modification of Munoz and Becker(13) was employed. The serum agar contained 1 part of rabbit antiserum diluted 1:5, 1 part of 0.6% agar which had been previously adjusted to pH 7.5 and clarified in 0.15 M NaCl. After cooling, 0.4 ml of the antigenic solution was overlayed on the agar. Several dilutions of the C-RP solution in 0.15 M NaCl were used to determine the rate of penetration of the precipitating bands. The serial dilutions of this antigenic solution contained respectively 0.080, 0.040, 0.020, 0.010, 0.005 mg of crystalline C-RP per ml. *Agar precipitin (Ouchterlony) tech-*

* This investigation was aided in part by a grant from the Asthmatic Children's Aid and Schieffelin and Co.

† Research Fellow, Allergy Research Unit, Chicago Medical School.

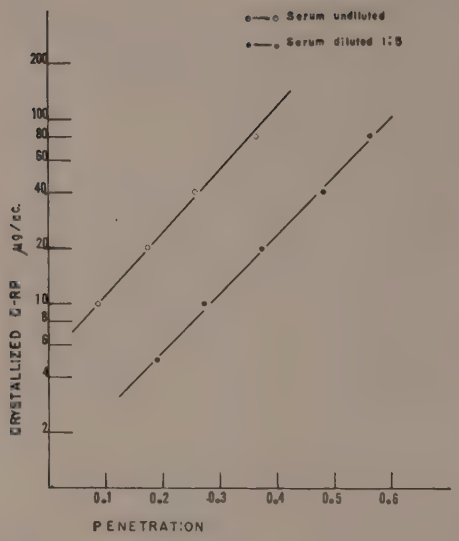

FIG. 1. Penetration values of serial dilutions of crystalline C-reactive protein into agar-serum. Serum contained 0.625 mg of antibody N/ml. Penetration values computed from formula: Penetration (P) = Distance (D)/$\sqrt{\text{Time (T)}}$.

nic—The Ouchterlony technic was used as modified by Halbert *et al.*(15). 0.15 ml of rabbit antiserum was placed in the central well, and 0.1 ml of patients sera in the peripheral wells. The plates were then placed in the refrigerator at 6°C and the precipitin bands read at intervals starting from 7 days. The final readings were done after 24 days.

Results. Crystalline C-RP obtained from the thoracocentesis fluid of a patient with Hodgkin's disease, showed precipitin bands migrating in antiserum-agar medium. The migration of the bands followed a straight line relationship with the square root of the time. The rate of penetration appeared to be directly proportional to the log of C-RP concentration and inversely proportional to the antibody content of the rabbit antiserum. Fig. 1 was obtained by plotting the penetration against the concentrations of crystalline C-RP (respectively, 0.08, 0.04, 0.02, 0.01, 0.005 mg/ml). The rabbit antiserum contained 0.652 mg of antibody nitrogen per cc and was used diluted 1:5 and undiluted.

With antisera containing a known concentration of antibodies, the penetration rates of the C-RP present in the blood of 16 patients with rheumatic fever were determined. The rates of penetration ranged between 0.652 and 0.100. The concentration of the protein was calculated by the use of the standard curve of penetration of the crystalline C-RP (Fig. 1), and was expressed in terms of mg of protein per cc of serum. The values ranged between 0.140 and 0.003 mg ml.

We further studied the immunological reactions between C-RP and C-RP antiserum by the technic devised by Ouchterlony(16). When crystalline C-RP was allowed to diffuse against rabbit C-RP antiserum, 3 distinct bands appeared, one sharp and 2 faint. Increasing the concentration of the crystalline C-RP increased the distance between the precipitation bands and the antigen wells; but no variations in the shape of the lines of precipitation were noted. The sera of 71 pa-

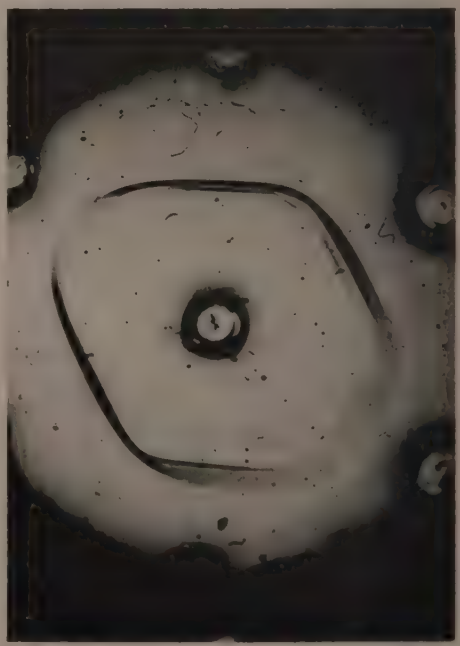

FIG. 2. Agar-serum precipitation bands with sera of patients with acute rheumatic fever tested against rabbit anti-crystalline C-RP serum. Undiluted rabbit antiserum in the central well. Human sera in the peripheral wells. Photographed after 24 days at 4°C.

tients with different diseases were studied with the Ouchterlony technic. From 0 to 3 bands of precipitation were formed in the agar medium. Sometimes it was difficult to define the exact number of the formed bands. However, more than 3 precipitation bands were never observed. When 2 or 3 lines were noted, one was normally quite intense and common to all the sera; others were moderately to very delicate. The sharpness of the bands also showed considerable differences. Some were clearly defined, others faint and diffuse. The heaviest common line was apparent first; the others took several more days to appear. The final configuration of the bands was present after 3 weeks (Fig. 2). No relationship was observed between the concentration of the C-RP in the blood (as determined by the penetration rate of the main band in the Oudin tubes) and the number and shape of the precipitation lines present in the agar plates.

Several controls confirmed that no cross reactions occurred between human normal sera and the rabbit C-RP antisera used. No bands appeared when sera containing high levels of C-RP were allowed to diffuse against pneumococcus C-polysaccharide.

Discussion. The total amount of C-RP that is present in the "acute phase serum" is very important in evaluating an inflammatory process. It is most helpful in those cases in which the concentration of the C-RP in the serum is low, and when other laboratory tests and clinical evidence of activity are equivocal (4,14). The use of the agar diffusion technic offers the possibility of evaluating with a high degree of accuracy very small amounts of the C-RP present in the serum.

Each precipitin band represents an antigen-antibody system (16-17), therefore, at least 3 antigens are present in the C-RP in its crystalline form. These 3 antigens are present in "acute phase sera" in different concentrations. The quantity of one antigen is not apparently related to the amount of the others. The possibility has been suggested by Jennings (18) that multiple bands represent several determinant groups of a single antigen. From the data reported here, both of these possibilities are likely.

The results obtained indicate that an inflammatory stimulus causes the appearance in the blood stream of varying amounts of C-RP which may give rise to differences in its antigenic components. Determination of the values of these components may be of importance in studying the progress of an inflammatory process.

Summary. 1. The rate of penetration of crystallized C-RP into agar-antiserum medium has been determined. 2. With this procedure, the C-reactive protein present in human serum of 16 patients with rheumatic fever was evaluated. Variations of less than 0.001 mg of C-RP per ml of serum were detectable. 3. By observing the pattern of the precipitin bands at least 3 antigenic components were found to be present in crystalline C-reactive protein. 4. In human serum, the C-RP present appeared to contain up to 3 antigenic components in different concentrations.

1. Abernathy, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, v73, 173.

2. McCarty, M., *ibid.*, 1947, v85, 491.

3. MacLeod, C. M., and Avery, O. T., *ibid.*, 1941, v73, 183.

4. Anderson, H. C., and McCarty, M., *Am. J. Med.*, 1950, v8, 445.

5. Hill, Alan G. S., *Lancet*, 1952, v2, 558.

6. Stollerman, G. H., Glick, S., Patel, D. J., and Hirshfield, I., *Am. J. Med.*, 1953, v15, 645.

7. Shetlar, M. R., Bullock, J. A., Shetlar, C. L., and Payne, R. W., PROC. SOC. EXP. BIOL. AND MED, 1955, v88, 107.

8. Kroop, I. G., and Shackman, N. H., *ibid.*, 1954, v86, 95.

9. Weiner, H. E., and Moshin, J. R., *Am. Rev. Tuberc.*, 1953, v68, 594.

10. Freund, J., *Ann. Rev. Microbiol.*, 1947, v1, 291.

11. Wood, H. F., McCarty, M., and Slater, R. J., *J. Exp. Med.*, 1954, v100, 71.

12. Kabat, E. A., and Mayer, M. M. *Experimental Immunochemistry*. Charles C. Thomas, Springfield, Ill., 1948.

13. Munoz, J., and Becker, E. E., *J. Immunol.*, 1950, v65, 57.

14. Wood, H. F., and McCarty, M., *J. Clin. Invest.*, 1951, v30, 616.

15. Halbert, S. P., Swick, L., and Sonn, C., *J. Exp.*

Med., 1955, v101, 557.
16. Ouchterlony, O., *Acta Path. et Microbiol. Scand.*, 1949, v26, 507.
17. Bjorklund, B., *Internat. Arch. Allergy & Appl. Immunol.*, 1953, v4, 340.
18. Jennings, R. K., *J. Bact.*, 1954, v67, 565.

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Triazenes as Inhibitors of Mouse Sarcoma 180.* (22073)

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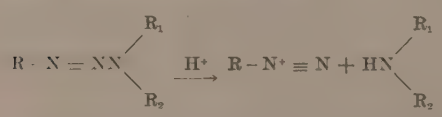
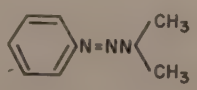
3,3-Dimethyl-l-phenyltriazene (Fig. 1) has recently been found to inhibit *in vivo* growth of the mouse sarcoma 180 (S-180). Tumor inhibitory activity has not previously been described for triazenes; thus a new biological activity is disclosed for derivatives containing the diazo moiety to which this activity might be ascribed. The triazenes are characterized by a high degree of lability in the presence of H-ions, resulting in the formation of a diazonium ion and an amine, thus:



The vigorous coupling activity of the diazonium ion is well known and this particular property of the triazenes ("diazoamines") has been utilized to advantage in the synthesis of azo dyes(1), for polymerization in the rubber industry(2), and for alkylation of dissimilar hydrocarbons in the synthesis of high-octane gasoline(3). It has heretofore been demonstrated that S-180 is sensitive to alkylating agents such as the mustard gases(4), triethylene melamine(5), and the polyfunctional phosphoramides(6). It therefore seemed reasonable to assume that a compound such as 3,3-dimethyl-l-phenyltriazene (hereafter to be referred to as "the triazene") could achieve systemic distribution intact and, at the cellular level, be subject to H-ion catalysis with liberation of benzenediazonium ion and dimethyl amine. The diazonium ion might then inhibit S-180 by reacting with elements essential for cell proliferation—a phenomenon postulated for other alkylating agents(7). This hypothesis will be examined below. It remains to be seen whether the triazenes are sufficiently selective for tumor cells over normal, rapidly proliferating tissue to be useful in higher mammals. In the mouse the therapeutic index is fairly low.



3,3-DIMETHYL-1-PHENYLTRIAZENE

FIG. 1.

Special emphasis will be given the triazene described above since it has proved so far to be the most interesting member of the series (Table I). A study of other diazoamines is continuing in an effort to establish a correlation between structure and tumor-inhibitory activity.

Materials and methods. Dimethyl-phenyltriazene† was synthesized by one of us (C. S. R., Jr.)(8). The compound is a pale, amber fluid with a specific gravity of 1.02.

* This investigation was supported in part by an institutional research grant from the American Cancer Society, by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of National Research Council and by a grant from the National Cancer Institute, U. S. Public Health Service.

TABLE I. Triazene Analogs Which Have Been Adequately Tested for S-180 Inhibition.

Compound	Source
Positive*	
3,3-Dimethyl-l-phenyltriazene	Dr. C. S. Rondestvedt, Jr. (U. Mich.)
3,3-Dimethyl-l-p-nitrophenyltriazene	"
3,3-Dimethyl-l-p-tolyltriazene	"
5(l-Naphthylazo) guanylurea	American Cyanamid Co.
1(5-Chloro-2-tolylazo)-5-(4-methoxy-2-sulfophenyl) biguanide	"
Negative	
3,3-Diethyl-l-phenyltriazene	Dr. R. K. Barclay (Sloan-Kettering)
Diazoaminobenzene	"
4,4′-(3,3′-Dimethoxy-4,4′-biphenylenebisazo)-morpholine	Eastman Kodak Co.
(3,3′-Dimethoxy-4,4′-biphenylene) bis-3,3-dimethyltriazene	"
1-[3,3-bis(2-Cyanoethyl)triazeno]-anthraquinone	"
1-(3,3-Dimethyltriazeno)-anthraquinone	"
1,1′-(4,4′-Biphenylene)bis(3,3-dimethyltriazene)	Parke, Davis & Co.
N^1-Phenylazo-N^2-phenylurea	Dr. Karl Pfister (Merck & Co.)
1-p-Nitrophenyl-3-phenyltetrazene	Remington Rand
1-p-Carboxyphenyl-3-phenyltetrazene	"
4-[3,3-bis(2-Hydroxyethyl)triazeno]benzenesulfonamide	Chem.-Bio. Coordination Center
1,3-di(4-Methoxyphenyl)triazene	Confidential
Diazo-l-amino-2-naphthol-4-sulfonic acid	National Aniline Co.
2,2′-Diazoaminofluorene	Dr. F. E. Ray (U. Florida)
4-(p-Nitrobenzenediazoamino)azobenzene	Purchased

* At tolerated doses tumor avg diameters of treated mice were 75% or less of the avg diameters of control tumors.

It is characterized by a pungent, aromatic odor and is irritating to mucous membrane when applied undiluted. Uniformity and stability of the various samples used were evaluated by ultraviolet spectrophotometry. The triazene is stable in alkali, but is rapidly decomposed by acid. It is, however, but sparingly soluble in water. For tumor inhibition studies, 18-22 g Swiss albino mice (Millerton Research Farms) were employed. Except where possible sex differences were to be explored, it was customary to use female mice. Single trocar implants of S-180 weighing *ca* 5 mg (wet weight) were made subcutaneously in the right axillary region. The implants were cut from tissue excised from donor mice after 7 days of growth. Usually therapy was initiated by direct oral intubation or by intraperitoneal injection 24 hours post-implantation and was continued for 7 successive days. Preliminary experiments established that it was immaterial whether the daily dose was given at one time or divided equally and the 2 half doses given at 7-hour intervals. Therefore the daily dose, uniformly contained in 0.1 ml of peanut oil/20 g mouse, was given as a single injection. Control mice received peanut oil alone by the appropriate route. All animals were weighed on the day of implantation and the day following the last injection. Tumors were measured through the skin with calipers. Two perpendicular diameters were determined and the mean obtained for the purpose of expressing tumor size as an "average diameter." In some experiments, the mice were sacrificed immediately thereafter and the tumors excised and weighed for the purpose of demonstrating the relationship between mass and average diameter.

† Original sample was supplied through the Chemical-Biological Coordination Center, Washington, D. C., as also was the case with 3,3-dimethyl-l-p-nitrophenyltriazene.

Results and discussion. Intraperitoneal doses of the triazene employed in this study

TABLE II. 3,3-Dimethyl-l-phenyltriazene Inhibition of S-180. Treatment given intraperitoneally and continued for 7 days.*

Daily dose, mm^3/kg	Avg wt change, g	Avg tumor diameter, mm	Avg tumor wt (wet) mg
62.5	−3	7.0	89.5
31.25	0	7.9	153.6
15.63	+1	9.3	234.2
0.00	+2	10.8	389.5

* Composite data from 3 separate experiments; a total of 60 mice/dose level.

TABLE III. 3,3-Dimethyl-1-phenyltriazene Inhibition of S-180 and Toxicity to Host following Oral Intubation. Therapy continued for 7 days.

Daily dose, mm³/kg	Avg tumor diameter, mm	Toxicity: Avg wt change, g	Toxicity: Mortality
250	6.4*	−5.3*	16/20
125	6.4	−4.0	0/20
62.5	8.5	−2.0	"
0.0	12.0	−1.0	"

* Based on 4 surviving animals only.

have ranged between 62.5 and 15.63 cm/kg/day; with these 2 extremes and an intermediate dose of 31.25 cm, a 3-point curve may be constructed from wet weight data (Table II) which is a straight-line function when the *log* of the dose is plotted against per cent inhibition of tumor growth. Thus, a linear dose-response relationship exists between the inhibitor and its effects on S-180 growth with a 50% effective dose of approximately 22 cm; this was found to be the case, irrespective of sex of the host. Table II also shows the relationship between "average diameter" and the actual mass of tumor tissue. In the tumor-bearing Swiss mouse, repeated daily doses of the triazene greater than 62.5 cm/kg proved lethal to most animals prior to completion of a 7-day course (Table III). Indeed, the 62.5 cm regimen resulted in delayed lethality not attributable to tumor growth (Table IV).

The above tolerated dosage schedule of the triazene was also used to treat animals with "established" tumors. Intraperitoneal therapy was begun 96 hours after tumor implantation and was continued for 7 days thereafter. The relative growth retardation produced by each dose level was comparable with that seen when therapy was started at the earlier post-implantation interval.

Since the triazene is acid-labile it was anticipated the agent would prove ineffective when administered by the oral route unless precautions were taken to neutralize gastric juice. The triazene, however, not only produced tumor-growth inhibition but also host intoxication in the same order of magnitude of dose when administered either orally or by intraperitoneal injection (Table III). Other than dissolving in peanut oil, no precautions were taken to protect the compound from attack by gastric acidity.

S-180 grew, but failed to resume *rapid* growth, following an inhibitory course of the triazene. This observation suggested viability of tumor cells, or their proliferative capacity, had been seriously impaired by the agent. Gross examination of treated tumors revealed a firm, compact consistency with no evidence of extensive necrosis, suggesting that necrotizing activity by the agent was not responsible for sluggish growth. To explore this point further, a series of mice implanted with S-180 was treated by intraperitoneal injection for 7 days with 62.5 cm/kg/day of the triazene. Pieces from treated and control tumors were implanted into normal mice the day following the last injection. One week later the treated S-180 had attained an average diameter of 4.7 mm whereas untreated tumor had grown to an average diameter of 12.8 mm (20 mice/group). There were no instances of failure to "take" among the reimplanted, treated tumors which indicated that some, if perhaps not all, cells remained viable.

Alkylating agents such as nitrogen mustard and triethylene melamine (TEM) characteristically cause depletion of the cellular elements of the marrow in intoxicated animals (9). Since there existed a reasonable expec-

TABLE IV. Relationship between S-180 Inhibitory Doses and Delayed Lethality to Host of 3,3-Dimethyl-1-phenyltriazene. Seven successive days of intraperitoneal administration.

Daily dose, mm³/kg	Tumor-bearing mice: End of therapy: Avg tumor diam., mm	Tumor-bearing mice: End of therapy: Mortality	Tumor-bearing mice: Mortality 7 days later	Non-tumor-bearing mice: End of therapy mortality	Non-tumor-bearing mice: Mortality 7 days later
62.5	7.2	1/30	16/30	0/30	11/30
31.25	8.9	0/30	1/30	"	0/30
15.63	11.8	1/29	9/29	"	2/30
0	13.5	0/30	3/30	"	0/30

TABLE V. Effects of Nicotinamide and Nicotinic Acid on Activity of 3,3-Dimethyl-1-phenyltriazene in S-180-Bearing Mice. Therapy was given I.P. for 7 days; uniformly, when combined, the vitamin derivatives preceded the triazene.

Agent and dose/kg/day	Avg diam. of tumors, mm	Avg wt change, g	Mortality
Triazene, 100 mm³	—	—	10/10
" , 50 "	10.1	−4.5	2/10
Nicotinamide, 500 mg	14.8	+ .5	0/10
" " + triazene, 100 mm³	—	—	10/10
" " + " 50 "	8.3	−2.5	0/10
Nicotinic acid, 500 mg*	15.5	.0	"
" " + triazene, 100 mm³	7.8	−4.5	3/10
" " + " 50 "	10.1	−2.0	0/10
Control	15.4	.0	"

* Prepared as the sodium salt immediately prior to injection.

tation that the biological activity of the triazene might reflect the alkylating properties of the benzenediazonium ion, experiments were undertaken to learn if marrow depression might characterize triazene intoxication. Groups of non-tumor-bearing mice were treated with intoxicating doses of TEM (1 mg/kg/day) and the triazene (62.5 cm/kg/day) for 5 days. Another group, serving as control mice, received daily injections of peanut oil, the vehicle used for the 2 agents. Although the triazene-treated mice lost more weight (avg −3.1 g) than did those receiving TEM (avg −1.6 g), the total femoral nucleated cell count for the TEM-treated mice was 33% of the control count, whereas the count for triazene-treated mice was 50% of control values.‡ Thus, the triazene did depress bone marrow and this, undoubtedly, contributes to the overall intoxication of the mouse. However, it is distinctly less potent in this respect than is TEM under the conditions of this experiment.

Phillips(10) observed that mice, intoxicated with the triazene, developed a syndrome characterized by unsteady stance and gait, choreaform movements of the head, and retropulsion (the "waltzing" syndrome). The behavior pattern so closely resembled that described by Goldin *et al.*(11) for mice intoxicated with certain alkylating agents that the "swimming test" applied by the latter investigators to detect the presence of this neurological disturbance was applied in this instance. The results were positive. In separate studies, Dagg *et al.*(12) demonstrated a teratogenic response to the triazene in the developing chick embryo which could be prevented by the administration of nicotinamide. The effects of nicotinic acid or its amide were therefore examined in tumor-bearing mice treated with the triazene. The results of a typical experiment are presented in Table V. It is evident tumor-inhibitory activity of the triazene was not altered by prior administration of nicotinic acid or the amide. However, mice treated with nicotinic acid and surviving the 100 cm triazene dose failed to exhibit the "waltzing" syndrome. In other experiments, 2 groups of non-tumor-bearing mice were treated with nicotinic acid (500 mg/kg/day, as the sodium salt) for 3 consecutive days, and a third group received control injections of isotonic NaCl (10 mice/group). One hour after the last intraperitoneal injection, the group injected with NaCl and one of the groups which had received sodium nicotinate received a single injection of the triazene. The dose of the triazene used (180 cm/kg) was one which previously had been shown capable of inducing the swimming deficiency mentioned above within 24 hours in the majority of mice without subsequent lethality. Examined the day following the triazene injection, 70% of the mice which had received isotonic NaCl and the triazene showed the

‡ The method of quantitating total nucleated cells of mouse femurs has been perfected at Sloan-Kettering Institute by Dr. Vincenzo Grifoni, a Visiting Fellow from the U. of Milan, Italy. Details of the method and some of its applications will be the subject of a future publication.

swimming defect (as well as the behavior symptoms of the "waltzing" syndrome) but none of those mice pretreated with nicotinic acid were so affected. There was no change in this situation for the following 3 days of observation. Any relationship between induction of teratogeny and induction of the "waltzing" syndrome by the triazene, other than that both abnormalities may be prevented by nicotinic acid derivatives, is obscure.

Consideration was given the possibility that chemical interaction in the peritoneal cavity between nicotinic acid and the triazene might account for the alteration in biological activity of the triazene. Appearing to negate this possibility were the results of an experiment wherein the nicotinic acid derivatives were administered by oral intubation 45 minutes prior to intraperitoneal injections of the triazene. Doses of agents were similar to those described in Table V and the results were essentially identical. Or, in another experiment with similar end-results, the vitamin derivatives were injected intraperitoneally 60 minutes before doses of 100, 75, and 50 cm/kg/day of the triazene. Finally, *in vitro* experiments were conducted wherein dilute solutions of nicotinic acid and its amide were added to buffered (pH 6.8) aqueous solutions of the triazene. These preparations were examined for possible changes in the ultraviolet absorption patterns. No significant changes were detected, suggesting failure of interaction under these conditions.

It may be adduced from the above that one of the features contributing to intoxication of the Swiss mouse by the triazene is a central nervous system lesion, the overt manifestations of which correspond with those of the genetically recessive characteristics described for Waltzer or Shaker mice(13). These manifestations may be prevented by the prior administration of nicotinic acid. The precise identity of this lesion with that described by Goldin *et al.*(11) remains to be established. Nevertheless, there is similarity between the circumstances leading to development of the lesion under the 2 experimental conditions which tends to support the notion that the triazene, as was found by Golden *et al.* for specific chlorinated tertiary amines, exerts a part of its biological activity by presenting the mouse with 1) an essential structural configuration and 2) an alkylating group. Failure to protect against triazene-induced tumor inhibition with nicotinic acid, however, suggests a bifunctional capacity of the triazene or, possibly a triazene-catabolite, farther removed from the diazonium ion, possessing tumor-inhibitory properties. Indeed, it is clear from the data in Table V that the higher doses of triazene were lethal to some individuals despite the administration of nicotinic acid but none of those animals, though obviously ill, demonstrated overt manifestations of the central lesion prior to terminus; this is further support for perceiving separable activities for the triazene.

Summary. 1. 3,3-Dimethyl-l-phenyltriazene possesses inhibitory activity for growth of the mouse tumor, sarcoma 180. Certain other triazenes have similar activity. 2. Dimethyl-phenyltriazene produces comparable tumor inhibition whether administered orally or intraperitoneally. 3. The triazene induces the "waltzing" syndrome in the Swiss albino mouse used in this study. 4. Nicotinic acid administered prior to the triazene prevents the "waltzing" syndrome but does not modify tumor inhibition. 5. In doses intoxicating to the Swiss mouse, the triazene is a moderate bone marrow depressant.

1. German patent 513,209 to I. G. Farbenind. A.-G., 7.15.28.

2. Levi, T. G., *Gomma,* 1937, v1, 4.

3. Dutch patent 60,768 to N.V. de Bataafsche Petroleum Maatschapij, 3.15.48.

4. Shapiro, D. M., Goldin, A., Landing, B. H., Bergner, A. D., Faiman, F., and Goldberg, B., *Cancer,* 1949, v2, 100.

5. Buckley, S. M., Stock, C. C., Crossley, M. L., and Rhoads, C. P., *ibid.,* 1952, v5, 144.

6. Buckley, S. M., Stock, C. C., Parker, R. P., Crossley, M. L., Kuh, E., and Seeger, D. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 299.

7. Elmore, D. T., Gulland, J. M., Jordan, D. O., and Taylor, H. F. W., *Biochem. J.,* 1948, v42, 308.

8. Elks, J., and Hey, D. H., *J. Chem. Soc.,* 1943, 441.

9. Philips, F. S., and Thiersch, J. B., *J. Pharmacol. and Exp. Therap.,* 1950, v100, 398.

10. Philips, F. S., to be published.

11. Goldin, A., Noe, H. A., Landing, B. H., Shapiro, D. M., and Goldberg, B., *J. Pharmacol. and Exp. Therap.*, 1948, v94, 249.

12. Dagg, C. P., Karnofsky, D. A., Stock, C. C., Lacon, C. R., and Roddy, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 489.

13. Grüneberg, H., *The Genetics of the Mouse*, 2nd Ed. Martinus Nijhoff, The Hague, Netherlands, 1952, p178ff.

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Effects of Certain Triazenes on Chick Embryos and on Tumors Explanted to the Chorioallantois.* (22074)

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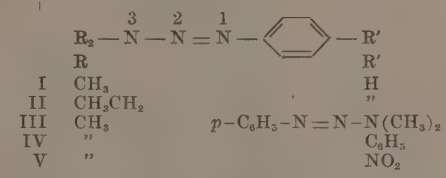
During the screening of various chemical agents for chemotherapeutic activity against the mouse sarcoma 180, it was found that 3, 3-dimethyl-1-phenyltriazene significantly inhibited the growth of this tumor in mice(1). On the basis of the preliminary results against the sarcoma 180, this agent and several related compounds were selected for more extensive study in secondary systems used in the tumor screening program(2-4).

This report is concerned with the effects of certain triazenes on the development of the chick embryo and on tumors growing on the chorioallantois. In the studies reported here it was found that the anomalies induced in the chick embryo by triazenes were similar to those caused by other, unrelated, teratogens whose effects are preventable or reversible by nicotinamide(5-9). Consequently, nicotinamide was tested for its ability to prevent the teratogenic effects of the triazenes.

Materials and methods. Commercially obtained White Leghorn eggs, incubated at 38°, were used for all experiments. The compounds tested for teratogenic activity against the chick embryo have the following structures:



	R	R'
I	CH_3	H
II	CH_3CH_2	"
III	CH_3	p-C_6H_5-N=N-N$(CH_3)_2$
IV	"	C_6H_5
V	"	NO_2

I: 3,3-dimethyl-l-phenyltriazene
II: 3,3-diethyl-l-phenyltriazene
III: 1,1'-(4-4'-biphenylene) bis [3,3-dimethyltriazene]
IV: 1-(4-biphenylyl)-3,3-dimethyltriazene
V: 3,3-dimethyl-l-*p*-nitrophenyltriazene

I is an amber-colored liquid with a specific gravity of 1.02. II is also a liquid, while III, IV, and V are solids. Because of their low solubility, these compounds were prepared as suspensions in isotonic saline or in 0.5% sodium carboxymethylcellulose in isotonic saline. The freshly prepared suspensions were injected into the yolk sac of 4-day-old embryos. The eggs were sacrificed after the 18th day of incubation. The embryos were weighed, examined for gross abnormalities, and selected embryos were cleared and stained with alizarin to show skeletal defects. In the protection experiments, nicotinamide in isotonic saline was injected immediately following the triazene. The tumors used were mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3 (H.Ep.#3)(10,11). Preparatory to chemotherapy trials, triazene-I was injected into the yolk sac of 12-day-old embryos to determine tolerated dose levels

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PLATE I

FIG. 1. 18-day control embryo.
FIG. 2. 18-day embryo treated with triazene-I at 4 days.
FIG. 3. Skeleton of 18-day control embryo.
FIG. 4. Skeleton of 18-day embryo treated with triazene-I at 4 days.

and the morphological alterations in the surviving embryos.

Single fragments of tumor were explanted to the chorioallantois on the 8th day of incubation. Four days later, after those eggs with implanted and apparently viable tumors had been selected, triazene-I was injected into the yolk sac. On the 16th day of incubation the eggs were sacrificed; the tumors were weighed and portions saved for microscopic examination. Whenever possible the tumors were bioassayed. Fragments of the H.Ep.#3 were pooled in 100-150 mg lots in each experimental group and inoculated into irradiated-cortisonized rats. Individual tumors of the S180 were assayed by subcutaneous implantation into mice.

Results. Teratogenic activity of 3,3-dimethyl-1-phenyltriazene: Triazene-I, when injected on the 4th day of incubation, produced consistent and characteristic abnormalities in development, clearly seen in embryos surviving to the 18th day of incubation (Fig. 1-4). The LD_{50} was 0.5-1.0 cmm egg and the teratogenic effects were produced over this range (Table I). The abnormal embryos showed weight inhibition associated with skeletal deformities of the beak and appendages. Edema and feather growth inhibition, with clubbing, occasionally occurred.

TABLE I. Toxicity and Incidence of Abnormal Embryos in Eggs Injected with Triazenes on 4th Day of Incubation and Sacrificed after 18th Day. Protection against teratogenic effects of triazenes with nicotinamide.

	Dose, mm^3/egg	No. inj.	Mortality, %	Abnormal survivors, %
T*-I	.25	20	40	25
	.5	47	30	79
	1.0	25	76	100
	2.0	15	93	100
T-II	.25	20	35	69
	.5	45	49	100
	1.0	20	45	100
	2.0	15	67	100
T-I + N*	.5 / 5.0	45	62	0
T-II + N	.5 / 5.0	43	86	0
T-II + N	.5 / 1.25	49	78	0

* T = Triazene; N = Nicotinamide.

The principal beak defect was parrot-beak (Fig. 2 and 4), although less severe effects such as shortening of the upper and lower beak, or the lower beak alone, were sometimes seen. Abnormalities of the appendicular skeleton included micromelia, bending of the tibiotarsus, and occasionally, bending of the tarsometatarsus. No abnormalities were found in the axial skeleton or in the pelvic and shoulder girdles.

Following injections into the 12-day yolk sac, no gross skeletal abnormalities were produced at tolerated doses. Doses of 5 to 10 cmm/egg led to stunting, inhibition of feather growth with clubbing, and edema in a large percentage of the embryos. Another consistent effect in the 12-day embryo was a reduction in spleen weight. Thus, at 16 days the control spleens weighed 13.6 mg, on the average, as compared to 4.5 mg for the affected embryos.

Teratogenic activity of other triazenes: The tolerated doses and the estimated LD_{50}'s of triazenes II, III, IV, and V in the 4-day embryo did not appear to be appreciably different from triazene-I. In this series, only 3,3-dimethyl-1-p-nitrophenyltriazene failed to produce any abnormalities. Triazene-II induced the same pattern of embryonic malformations seen with triazene-I and at approximately the same doses. Triazene-III, however, was effective only at much higher dose levels and affected fewer of the surviving embryos. The overall teratogenic effects of triazene-IV were different from those of the other active compounds in that they occurred less consistently and the only abnormality observed was a bending of the tibiotarsus.

Protection with nicotinamide: Nicotinamide was tested against doses of triazene-I and II which, alone, produced a high incidence of abnormalities in the surviving embryos (Table I). Under these conditions nicotinamide protected against the teratogenic effects but did not restore the weight to normal nor did it protect against the lethality of triazene-I and II. The minimal effective ratio of nicotinamide to triazene has not been determined.

Tumor inhibiting effect of triazene-I: In the chemotherapy tests, triazene-I inhibited the growth of the S180 and H.Ep#3 (Table II). Histological sections of the tumor

TABLE II. Effect of Triazene-I on Mouse Sarcoma 180 and Toolan's H.Ep. #3 Growing on Chorioallantois of the Chick Embryo.

Group	Dose, mm^3/egg	No. inj.	No. survivors	Tumor wt, mg	Embryo wt, g
		Mouse sarcoma 180			
Control	0	8	7	172 ± 72	14.4 ± 1.9
Triazene-I	5.0	10	6	76 ± 44	12.9 ± 1.1
"	7.5	10	6	38 ± 39	10.0 ± 1.9
		Toolan's H.Ep. #3			
Control	0	29	26	229 ± 48	12.7 ± 1.3
Triazene-I	5.0	34	27	42 ± 30	10.0 ± 1.2
"	7.5	24	15	49 ± 37	9.1 ± .7
"	10.0	15	2	19 & 38	8.0 & 8.0

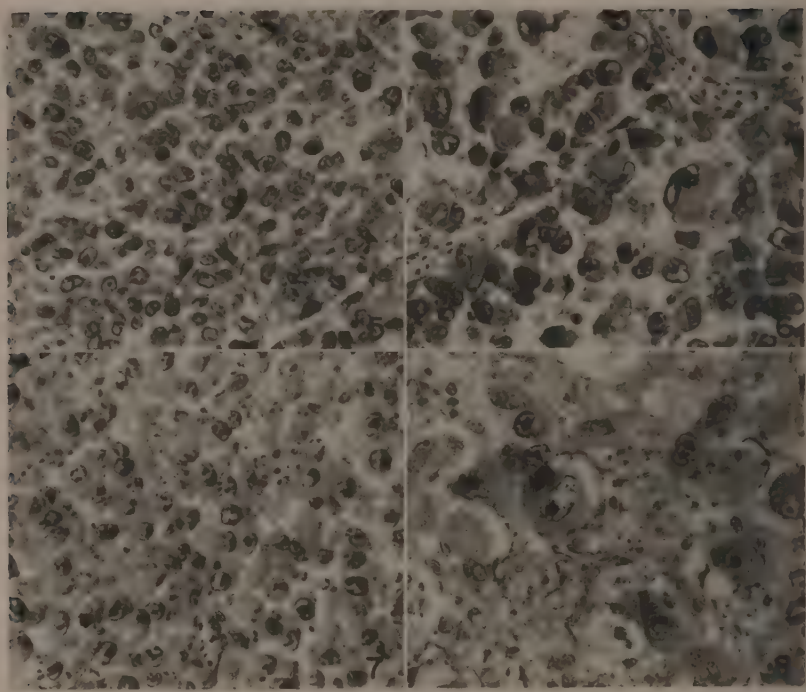

PLATE II

FIG. 5. Untreated mouse sarcoma 180 growing on the chorioallantois. × 200.

FIG. 6. Mouse sarcoma 180 growing on the chorioallantois, treated with triazene-I, 5.0 mm³ per egg. × 200.

FIG. 7. Untreated Toolan's H.Ep. #3 growing on the chorioallantois. × 200.

FIG. 8. Toolan's H.Ep. #3 growing on the chorioallantois treated with triazene-I, 7.5 mm³ per egg. × 200.

showed cellular enlargement, nuclear changes indicative of cellular injury, and a decrease in number of cells (Fig. 5-8). Histologically, the sarcoma 180 appeared to be more sensitive than the H.Ep.#3, since greater cellular damage was observed at the lower dose (5 cmm) in the sarcoma 180. When the sarcoma 180's were assayed in mice, the untreated tumors grew rapidly, but, in no case did any of the triazene-treated tumors show any evidence of growth during a 2-week observation period. This indicated that the triazene destroyed the viability of sarcoma 180 as measured by transplantation. In spite of the marked histological effects produced by triazene in the egg-grown H.Ep.#3, the tumors gave positive bioassays in rats. It is noted, however, that in order to prepare a satisfactory inoculum, several human tumors were pooled for bioassay, and therefore, the inclusion of a slightly damaged tumor in the pooled tumor inoculum could produce a positive bioassay. Thus, it is concluded that the viability of every one of the treated H.Ep. #3's was not destroyed by triazene.

Discussion. The effects of triazenes on chick embryos and on explanted tumors have interesting correlations with other agents. Of major significance is the observation that triazene-I causes distinctive and consistent effects on the mouse sarcoma 180 and Toolan's H.Ep.#3 explanted to the chorioallantois. The most important criteria of effect are the histological changes in the tumor and viability on bioassay. The histological changes of cellular enlargement and nuclear distortions with a decrease in cell numbers are similar to those engendered by nitrogen mus-

tard and related polyfunctional alkylating agents and by x-rays(12). The greater sensitivity of the sarcoma 180 as compared to H.Ep.#3 to triazene parallels the results seen with nitrogen mustard(11). A further similarity between the two types of compounds is that both the polyfunctional alkylating agents and triazene-I retard the growth of the embryonic spleen.

Quantitative dissimilarities between triazenes and polyfunctional alkylating agents are apparent in their comparative effects on 12-day-old chick embryos and egg-grown tumors. Doses of nitrogen mustard causing relatively slight disturbances in the embryo are markedly injurious to the tumors and affect their viability as indicated by bioassay (12,13). In contrast, the dose of triazene-I required to obtain an equivalent degree of tumor inhibition and cellular damage will induce definite injury to the embryo.

Despite certain similarities in the biological actions of nitrogen mustards and the triazenes, parallel information on the chemical reactivity of triazenes in biological systems is not available. It has been shown that among biologically active alkylating agents, compounds possessing 2 functional groups are far more active than the monofunctional agents (one-armed mustards)(14,15). While the amount of triazene necessary to produce effects in the explanted tumors in the egg are much greater than that required for most polyfunctional alkylating agents, 0.1 mg for nitrogen mustard as compared to 5 mg (5 cmm) for triazene-I, neither altering the structure of the triazenes (cf. triazene-I, II, IV, V) nor producing a two-armed compound (triazene-III) has enhanced the biological activity.

The triazenes are of further interest since certain members of this series produce a definite syndrome of malformations when injected into 4-day-old embryos. The effects on the facial and appendicular skeleton are similar, if not identical, to those following treatment by insulin(6,7), sulfanilamide (5,9), and eserine(5,8). Furthermore, as in the case of the latter compounds, nicotinamide will prevent the teratogenic action of triazenes in the 4-day embryo. This teratogenic property distinguishes triazenes from the polyfunctional alkylating agents which do not produce specific or consistent teratogenic effects. The biochemical mechanism for the production of the parrot beak-micromelia-bent tibiotarsus syndrome has not been definitely established, although Landauer(7) and Zwilling(9) have related it to interference with carbohydrate metabolism.

Since the effects of triazene-I on tumors and 12-day embryos are similar to those produced by certain polyfunctional alkylating agents while the effects on younger embryos resemble those following treatment with insulin, sulfanilamide and eserine, it is possible that the molecule is pluripotent or that it is converted in the egg into 2 or more metabolites with dissociated activity. In acid solutions triazene-I is broken to the diazonium salt and dimethylamine. Diazonium salts have been shown to polymerize *in vitro* to form diazoaminobenzene, a compound that is toxic to the liver, spleen, and kidneys in mice, and is carcinogenic in mice on local application to the skin(16). When injected into the 4-day-old embryo, *p*-diphenylamine diazonium fluoroborate produces a syndrome of abnormalities which is similar to that caused by triazenes-I and II(3).

Because of the wide range of biological effects of the polyfunctional alkylating agents, the discovery of a tumor-inhibiting drug with somewhat similar but also distinct effects, and acting by means of a considerably different chemical structure, becomes of interest.

Summary. 1. A new compound that inhibits the growth of the sarcoma 180 in mice, 3,3-dimethyl-1-phenyltriazene, "triazene," also exhibits teratogenic activity in the chick embryo and damages mouse and human tumors explanted to the chorioallantois of the chick embryo. 2. The teratogenic syndrome produced by the injection of "triazene" into the yolk sac of the 4-day embryo consists of parrot-beak, micromelia, bent tibiotarsus, edema, and feather growth inhibition. This effect can be prevented by the injection of nicotinamide. Several analogs of "triazene"

have been tested for teratogenic properties. 3. When injected into the yolk sac of embryos carrying chorioallantoic grafts of the mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3, "triazene" causes inhibition of tumor growth and cellular injury. This effect is similar to that produced by nitrogen mustard and other polyfunctional alkylating agents. Because "triazene" shows certain similarities in tumor-inhibiting activity when compared to nitrogen mustard and because of its distinctly different chemical structure, it warrants consideration as a candidate agent for clinical cancer chemotherapy.

We wish to express our appreciation to Dr. Christian S. Rondestvedt, Jr., of the University of Michigan for supplying several of the triazenes, and to Dr. Helene W. Toolan, of the Sloan-Kettering Institute for conducting the bioassays of the H.Ep. #3.

1. Clarke, D. A., Barclay, R. K., Stock, C. C., and Rondestvedt, C. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1955, in press.

2. Stock, C. C., *Adv. in Cancer Res.*, 1954, v2, 425.

3. Karnofsky, D. A., *Stanford Med. Bull.*, 1955, v13, 247.

4. Karnofsky, D. A., Ridgway, L. P., and Patterson, P. A., *Ann. N. Y. Acad. Sci.*, 1952, v55, 313.

5. Ancel, P., *La Chimiotératogenese. Réalisation des Monstruosites par des Substances Chimique chez les Vertébrés*, Paris (G. Doin), 1950.

6. Landauer, W., *J. Exp. Zool.*, 1947, v105, 145.

7. ———, *ibid.*, 1948, v109, 283.

8. ———, *Arch. Anat. Micros. et Morph. Exp.*, 1949, v38, 184.

9. Zwilling, E., and DeBell, J. T., *J. Exp. Zool.*, 1950, v115, 59.

10. Toolan, H. W., *Cancer Research*, 1954, v14, 660.

11. Dagg, C. P., Karnofsky, D. A., Toolan, H. W., and Roddy, J., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 223.

12. Stock, C. C., Biesele, J. J., Burchenal, J. H., Karnofsky, D. A., Moore, A. E., and Sugiura, K., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1360.

13. Karnofsky, D. A., Burchenal, J. H., Ormsbee, R. A., Cornman, I., and Rhoads, C. P., *A.A.A.S.*, 1947, 293.

14. Goldacre, R. J., Loveless, A., and Ross, W. C. J., *Nature*, 1949, v163, 667.

15. Biesele, J. J., Philips, F. S., Thiersch, J. B., Burchenal, J. H., Buckley, S. M., and Stock, C. C., *ibid.*, 1950, v166, 1112.

16. Kirby, A. H. M., *Cancer Research*, 1947, v7, 263.

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Serum Glycoprotein Concentrations in Rats Infected with the Cestode, *Hymenolepis diminuta*. (22075)

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(Introduced by Charles M. Carpenter.)

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The serum protein response in a large number of infectious diseases has been studied by many investigators employing electrophoretic or salting-out methods. Gutman(1), Fisher(2) and Stauber(3) have summarized the significant data in their reviews. In contrast, few reports are available on effects of infectious diseases on carbohydrate-containing proteins of serum. Studies concerned with the influence of infection on protein-bound carbohydrates of serum have been briefly reviewed by Weimer *et al.*(4). Diseases in which serum glycoproteins have been determined are characterized by invasion of tissues by the etiologic agent and marked, systemic host reactions. It was of interest to investigate the serum glycoproteins in an experimental infection in which the parasite is localized and invasion of the tissues does not occur.

The present investigation was undertaken to study the effects of experimental infection with *Hymenolepis diminuta* on serum concentrations of total glycoprotein, seromucoid (mucoprotein), γ-globulin polysaccharide, total protein, and γ-globulin protein in rats.

Hematocrit values and concentrations of hemoglobin were also determined in oxalated blood specimens.

Materials and methods. Twenty male and 7 female adult rats of the Long-Evans strain with an average weight of 305 g were infected. Thirteen male and 7 female rats served as controls. Tapeworm eggs were recovered from the feces of rats infected with *H. diminuta,* Rice Institute strain. Cysticercoids were obtained by feeding the eggs to laboratory-reared beetles, *Tenebrio molitor.* The rats were lightly anesthetized with ether and 5 cysticercoids were administered *per os* with a pipette. The animals were housed 2 per cage and were maintained on a diet of Purina Laboratory Chow and tap water, *ad libitum.* Thirty-two days after inoculation the infected rats were sacrificed by exsanguination from the heart and air embolism. The animals were autopsied and the tapeworms counted. Blood samples were obtained from the normal animals by cardiac puncture. Two ml of each of the blood specimens were added to tubes containing a dried mixture of potassium and ammonium oxalates. The remainder of the blood sample was allowed to clot and the serum separated. Total serum glycoprotein, seromucoid, γ-globulin polysaccharide, total serum protein, and γ-globulin protein were determined by methods previously reported(4,5). Hematocrit values were determined in Wintrobe tubes by the procedure outlined by Kolmer(6). Hemoglobin was determined by an acid-hematin method in a Klett-Summerson photoelectric colorimeter (6). The mean, standard error of the mean, t, and probability values were determined by standard statistical methods(7).

Results. A summary of the data is presented in Table I. No significant differences occurred between the normal and the infected group for the serum determinations. The increases in the hemoglobin and hematocrit values were significant at the 5% level.

The lack of effect of a well established infection with *H. diminuta* on the serum glycoproteins and proteins of the rat is in marked contrast to changes observed in other pathologic states of infectious etiology. The increase in hematocrit and in hemoglobin values might suggest a stimulation of the hemapoietic system, or reflect loss of water and plasma protein. Further studies are indicated.

TABLE I. Summary of Results.

Determination	Normal (20)	Infected (27)
Wt at bleeding (g)	343 ±14.0*	345 ±13.8*
No. of worms		3.9± .2
Total serum glycoprotein (mg %)	138 ± 2.0	143 ± 2.0
Seromucoid (mg %)	18 ± 1.2	18 ± .9
γ-globulin polysaccharide (mg %)	31 ± 1.7	30 ± .6
Total serum protein (g %)	6.6± .12	6.6± .9
γ-globulin protein (g %)	1.9± .06	1.9± .06
Hemoglobin (g %)	14.6± .4	16.1± .4
Hematocrit (%)	44.5± 1.2	47.7± .8

* Stand. error of the mean.

Several factors have been suggested as possible causes for the increased concentrations of serum glycoproteins in a wide variety of pathologic and physiologic states: tissue degradation(8), tissue proliferation and repair(9), protein synthesis(10), and nonspecific stress(11). Regardless of the cause of elevated serum glycoprotein levels, it is apparent that none of the responsible factors was operative in the present investigation.

It may be inferred from the results reported above, that a localized infection without tissue invasion has no significant effect on the level of the serum glycoproteins and proteins of the rat.

Summary. The effects of experimental infection with *Hymenolepis diminuta* on the serum concentrations of total glycoprotein, seromucoid, γ-globulin polysaccharide, total protein, γ-globulin protein and on hemoglobin and hematocrit values have been determined in Long-Evans rats. No statistically significant differences occurred in serum values between the normal and the infected groups. An increase in hemoglobin and hematocrit values, significant at the 5% level, was observed in the infected group.

1. Gutman, A. B., *Advances in Protein Chem.,* v4, Academic Press Inc., New York, 1948, 156.
2. Fisher, B., *Am. J. Clin. Path.,* 1953, v23, 246.
3. Stauber, L. A., *Exp. Parasit.,* 1954, v3, 544.
4. Weimer, H. E., Boak, R. A., Carpenter, C. M.,

Redlich-Moshin, J., Drusch, H. E., and Miller, J. N., *J. Inf. Dis.*, 1955, v96, 19.

5. Weimer, H. E., and Moshin, J. R., *Am. Rev. Tuberc.*, 1953, v68, 594.

6. Kolmer, J. A., Spaulding, E. H., and Robinson, H. W., *Approved Laboratory Technic*, 5th edition, Appleton-Century-Crofts Inc., New York, 1951.

7. Snedecor, G. W., *Statistical Methods*, 4th edition, Iowa State College Press, Ames, Iowa, 1946.

8. Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, v26, 90.

9. Shetlar, M. R., Bryan, R. S., Foster, J. V., Shetlar, C. L., and Everett, M. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 294.

10. Werner, I., *Acta Physiol. Scand.*, 1949, v19, 27.

11. Boas, N. F., and Peterman, A. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 19.

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Role of Hyperlipemia in the Genesis of Hypercholesteremia.* (22076)

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It has long been known that an hypercholesteremia, either occurring clinically or induced experimentally, is usually accompanied by a rise in the concentration of plasma phospholipid and sometimes also of neutral fat. This frequently observed association of hypercholesteremia with hyperlipemia has not been investigated in respect to the possible *sequential* relationship of these multiple lipid rises. Instead it has been more or less assumed, in lieu of direct evidence, that the lipid rises observed in hypercholesteremia are merely associative phenomena. Recently, however, Allbrink, Man and Peters(1), noting the physical association of plasma cholesterol with neutral fat in chylomicra, have posed the question of whether an excess of plasma neutral fat might not dissolve additional cholesterol thus preventing its inclusion in soluble lipoprotein, leading thereby in some manner to a state of hypercholesteremia.

In view of this last possibility, it was decided to induce a sustained state of hyperlipemia in the rat by continuous parenteral injection of 2 types of lipid mixture (containing only neutral fat and lecithin) and then observe the possible changes in plasma cholesterol resulting therefrom. The results obtained in this study, as herein reported, indicate that a state of hypercholesteremia quickly follows the induction of a prolonged hyperlipemia.

Materials and methods. Male rats (Long Evans strain) 12-16 weeks of age were used in all of the experiments. (a) Production of sustained hyperlipemia was accomplished in rats by rapid intravenous administration of 3 ml of either of 2 different lipid emulsions (B† and Z‡) followed by *continuous* intravenous administration of the same emulsion at 0.9 to 1.2 ml/hour for 12 hours. This continuous intravenous administration to an unanesthetized rat was made possible by its enclosure in the Bollman type of cage(2). Prior to the experiment these rats had been placed for 7-15 days on a diet comprised of Purina Chow enriched with cholic acid (2%) and olive oil (2%). Each rat also received an additional 3 ml of olive oil by stomach tube at 72, 48 and 24 hours prior to the start of experiment. All food was withheld from rats for 24 hours immediately prior to and during the experi-

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† B lipid emulsion (kindly furnished by Don Baxter) is composed as follows: Sesame Oil, U.S.P. 10; Dextrose, U.S.P. 4.5; Purified Soya Bean Lecithin 0.65; Water, q.s. 100 ml. Lipid is present as finely divided particles of 1 μ or less in diameter.

‡ Z lipid emulsion (kindly furnished by D. B. Zilversmit of University of Tenn.) is composed as follows: Coconut Oil 10; Purified Soya Bean Lecithin 1.0; Dextrose 5.0; Water, q.s. 100 ml. Lipid particles are approximately the same diameter as in B emulsion.

ment. This pre-feeding regimen was adopted after preliminary studies had demonstrated that the usual rapid decline in hyperlipemia after a single injection of a fat emulsion could be slowed if the animal's fat absorption previously had been enhanced. Plasma samples obtained before, immediately after the initial injection of 3 ml of fat emulsion, and 12 hours after beginning of continuous intravenous injection, were analyzed for cholesterol(3) and phospholipid(4). The total lipid of the samples was analyzed by the method of Bragdon(5). Control animals were fed same regimen but received glucose solution (5%) intravenously in lieu of fat emulsion. (b) *Partial hepatectomy.* Three animals were prefed and later administered lipid emulsion as described above except that just prior to lipid administration, the rats were anesthetized with ether and a portion (approximately 2.7 g) of liver removed. (c) *Production of hyperlipemia by injection of protamine.* Freshly prepared protamine sulfate solution (salmine)§ solution (2 mg in 3 ml normal saline solution) was injected into a series of rats given 3 ml of olive oil just prior to injection and into a control series fasted for 24 hours. Both series were given additional intravenous injections of 2 mg of protamine at 3, 6 and 9 hours following initial injection. For additional control purposes, a third series of rats were given 3 ml of olive oil and injections of dextrose solution (5%) comparable in volume to the volumes injected in the first 2 series. Plasma samples obtained before and 12 hours after the initial injection were analyzed for cholesterol and total lipid. (d) *In vitro extraction of cholesterol from hypercholesteremic serum by lipid suspension.* Pooled hypercholesteremic but non-turbid serum (cholesterol concentration: 128 mg 100 ml) was obtained from bile ligated, cholate fed rats(6). A lipid suspension was obtained as follows: The 24-hour intestinal lymph of 5 rats fed 3 ml of olive oil was collected and submitted to ultracentrifugation (r.c.f. = 13,000-27,000) for 30 minutes. The resulting top creamy layer was removed and suspended in 5 ml of H_2O. This

§ Eli Lilly and Co. control No. 8367-571094 DH.

TABLE I. Total Lipid and Cholesterol Response of Intact and Partially Hepatectomized Rat to Continuous Injection of Lipid Emulsion.

Rats				Bloot plasma								
				Total lipid (mg/100 ml)			Phospholipid (mg/100 ml)			Plasma cholesterol (mg/100 ml)		
No.	Given	Avg wt (g)	Total amt inj. (ml)	A	B	C	A	B	C	A	B	C
10	Control rats, dextrose sol.	301 (282–314)	12.5 (11.8–13.3)	286 (147–286)	—	219 (185–263)	101 (90–124) ± 2.3	—	125 (107–142) ± 3.5	62 (52–76) ± 2.24	—	80 (69–96) ± 2.44
13	B fat	299 (235–368)	15.2 (13.8–16.4)	189 (131 292) ± 13.2	—	681 (500–958) ± 53.0	97 (81–114) ± 4.8	185 (93–310) ± 38	196 (120–271) ± 15.4	53 (35–78) ± 3.4	60 (48–80) ± 2.4	124 (80–195) ± 11.2
5	Z fat	318 (304–328)	15.2 (14.8–15.4)	177 (138–296)	1888	864 (604–1140)	121 (100–176)	198 (184 238)	251 (181–327)	58 (48–70)	62 (51–67)	128 (88–180)
3	Partial hepatectomized rats, B fat	310 (302–318)	14.6 (14.0–15.2)	190 (181–200)	—	562 (523–597)	—	—	—	50 (47–51)	54 (45–64)	69 (67–73)

A = Before inj. B = Immediately after initial inj. C = 12 hr after inj.
Values in parentheses = Range; ± values = Stand. error of the mean.

TABLE II. Total Lipid and Cholesterol Response of Intact Rat to Intermittent Injections of Protamine.

Rats			Blood plasma			
			Total lipid (mg/100 ml)		Plasma cholesterol (mg/100 ml)	
No.	Given	Avg wt (g)	A	B	A	B
11	Protamine	311 (292–340)	138 (121–152) ± 5.9	238 (175–324) ± 21.4	48 (33–65) ± 3.6	60 (38–77) ± 3.4
9	Protamine & olive oil	325 (300–344)	204 (151–273) ± 20.3	563 (263–1390) ± 66.0	56 (39–78) ± 4.0	119 (96–174) ± 7.9
9	Olive oil	310 (302–318)	218 (148–356) ± 22.4	287 (210–394) ± 54	50 (47–51) ± 3.8	69 (67–73) ± 7.4

A = Before inj. B = 12 hr after inj.
Values in parentheses = Range; ± values = Stand. error of mean.

latter suspension was considered to consist almost entirely of a mixture of chylomicronous lipids, relatively free of protein(7) and had a cholesterol concentration of 148 mg and a total lipid concentration of 4440 mg/100 ml. Two ml of the lipid suspension were mixed with 2 ml of hypercholesteremic serum, shaken at room temperature for 6 hours and then the mixture ultracentrifuged. The resulting top creamy layer (approximately 0.9 ml) was removed, suspended in 5 ml of water and analyzed for cholesterol. The lower layer also was similarly analyzed. For control purposes, the identical procedure was repeated substituting a solution of H_2O for the hypercholesteremic serum.

Results. As Table I indicates, a continuous state of hyperlipemia characterized by a rise in both total lipid and phospholipid content of rat's plasma, was obtained by continuous injection of either B or Z lipid emulsion. Induction of this hyperlipemia resulted in a marked rise in cholesterol content of plasma. Thus when hyperlipemia was induced by injection of B emulsion, the average plasma cholesterol content increased from 53 mg before to 124 mg/100 ml in 12 hours, a rise of 134%. Approximately similar results in respect to cholesterol were obtained following parenteral administration of Z emulsion (See Table I), although its injection led to a greater elevation of plasma lipid, possibly due to its greater phospholipid content. Continuous injection of dextrose solution into control animals did not elevate their total plasma lipid but a moderate rise in their plasma phospholipid (24%) and cholesterol (29%) occurred. These latter rises however cannot be ascribed to administration of dextrose solution because they invariably occur in fat fed rats following any type of experimental procedure (*e.g.*, routine bleeding for samples).

Induction of lipemia in partially hepatectomized rats did not lead to a significant rise in plasma cholesterol. This failure of cholesterol to rise in partial absence of the liver suggests that the excess cholesterol observed in plasma of above intact rats after induced lipemia came from a hepatic and not an intestinal source. Starvation of all intact animals 24 hours prior to experimental procedure also suggests a source other than the intestine of the excess cholesterol.

The results following injection of protamine also suggested that experimental induction of lipemia alone induces a cholesteremia. Thus (Table II) although *intermittent* injection of protamine into the fasted rat produced a possible moderate increase in total lipid, it did not induce marked hypercholesteremia. However when the same dosage of protamine was given to rats fed olive oil, a significant hyperlipemia occurred and a hypercholesteremia (Table II). These results are in agreement with those found by Szasz and Constantinides(8).

In vitro exposure of pooled hypercholesteremic serum to the suspension of intestinal lymph lipids resulted in a fall of cholesterol content of serum from 2.56 to 1.0 mg and a

corresponding rise in cholesterol content of chylomicron lipids from 2.4 to 3.4 mg. The cholesterol content of control chylomicron lipid mixture remained unchanged. In other words, the lipid mixture was capable of extracting over half of the cholesterol originally present in clear hypercholesteremic serum.

Discussion. Our findings indicate that hypercholesteremia resulted whenever a sufficient degree of sustained hyperlipemia was induced by the methods employed. Furthermore, it appears that this cholesterol rise will occur regardless of whether the hyperlipemia ensues, because too much lipid is presented by parenteral administration to the organism for its complete removal from blood, or because the organism is prevented by protamine injection from removing its usual amount of lipid from blood. Cholesterol thus retained also originates primarily in the liver. Apparently a portion of the cholesterol normally discharged into plasma and then later taken up again by the liver is "trapped" in the excess lipid circulating in plasma. Ability of a lipid suspension derived from intestinal lymph to extract cholesterol existing in soluble, ostensibly lipoprotein form(9) in hypercholesteremic serum further suggests the possibility of such "trapping." Apparently then lipid can dissolve cholesterol existing in plasma, as Allbrink, Man and Peters(1) have suggested, and this occurs even when cholesterol is present in soluble lipoprotein bondage.

While previously hypercholesteremia and hyperlipemia have been considered independent processes, the demonstration that a rise in plasma lipid quickly leads to hypercholesteremia suggests that it is necessary to consider the possibility that an excess rise of cholesterol in plasma (when attended by hyperlipemia) may be passively induced resultant of *primary* derangement in plasma lipids other than cholesterol. This induced hypercholesteremia could have been effected by accumulation in plasma of either neutral fat, phospholipid or both lipids present in the emulsions. Present studies are in progress to determine the magnitude of particular responsibility of each of these other lipid species.

Summary. Induction of hyperlipemia in rats by continuous intravenous injection of lipid mixtures containing only neutral fat and soya lecithin quickly led to hypercholesteremia. These results suggest that in hypercholesteremia associated with hyperlipemia, the primary derangement may lie not in the cholesterol but in some phase of the fat metabolism.

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1. Allbrink, M. J., Man, E. B., and Peters, J. P., *J. Clin. Invest.*, 1955, v34, 147.

2. Bollman, J. L., *J. Lab. and Clin. Med.*, 1948, v33, 1348.

3. Saifer, A., and Kammerer, D. F., *J. Biol. Chem.*, 1946, v164, 657.

4. Zilversmit, D. B., and Davis, A. K., *J. Lab. Clin. Med.*, 1950, v35, 155.

5. Bragdon, J. H., *J. Biol. Chem.*, 1951, v190, 513.

6. Friedman, M., and Byers, S. O., *Am. J. Physiol.*, 1952, v168, 292.

7. Van Eck, W. F., Peters, J. P., and Man, E. B., *Metabolism*, 1952, vI, 383.

8. Szasz, G., and Constantinides, P., *Arch. Int. Pharmacod.*, 1953, v93, 255.

9. Byers, S. O., Friedman, M., Biggs, M. W., and Gunning, B., *J. Exp. Med.*, 1953, v97, 511.

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Chlorpromazine and Anesthetic Agents on Tissue Non-Protein Sulfhydryl Compounds in Normothermic and Hypothermic Rats.* (22077)

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Previous work(1,2) has shown that cold and restraint lower the concentration of non-protein sulfhydryl compounds (NPSH). Adrenalin also decreases liver and kidney NPSH (3) and it was later observed that some anesthetics alter liver NPSH. To determine the effect of anesthetics and chlorpromazine on tissue NPSH and the effect of cold on tissue NPSH of anesthetized animals, the NPSH in liver and kidney was measured on normothermic and hypothermic rats.

Methods and materials. The 210 adult Sprague-Dawley female rats were divided into the following groups: 52 control animals; 18 animals were given chlorpromazine hydrochloride; 17 were anesthetized with ether; 14 with nitrous oxide, 16 with phenobarbital sodium (phenobarbital), 39 with pentobarbital sodium (nembutal), 38 with thiopental sodium (pentothal), and 16 with thiamylal sodium (surital). Each group of treated animals was divided into 2 categories. Approximately one-half of the animals in each group was maintained at normal body temperature while the remaining animals were exposed in a cold room (0°C ± 1°C) and body temperature fall was regulated at 5°C per hour. All exposures were 4 hours in duration so that the body temperature of the hypothermic animals was approximately 18°C at the time of sacrifice. Chlorpromazine was injected intraperitoneally. These animals were in very light anesthesia exhibiting some reflex response to deep pain. Animals exposed to gaseous anesthetics (ether and nitrous oxide) were placed in a large glass chamber and the anesthetics and oxygen admitted at a rate to keep animals in very light surgical anesthesia. If animals were more deeply anesthetized, they would not survive the 4-hour exposure. Animals anesthetized with the barbiturates were injected intraperitoneally with phenobarbital, 20 mg/kg; thiamylal, 25 mg/kg; pentobarbital, 70 mg/kg; thiopental, 75 mg/kg initially, and thereafter sufficient to keep the animal in a relaxed state. In the barbiturate anesthesia experiments, the animals were maintained in surgical plane of anesthesia. The animals were then sacrificed and the organs removed immediately and frozen in dry ice. The NPSH was measured by a modification of the method of Benesch and Benesch(4).

Results. As may be seen from Table I, all anesthetized animals experienced a drop in liver NPSH. In the groups anesthetized with phenobarbital, thiamylal and thiopental, there was a significantly greater drop in the hypothermic categories than in the normothermic categories. However, in the groups given chlorpromazine, ether, nitrous oxide, and pentobarbital, exposure to cold did not have the usual effect of further decreasing the liver NPSH.

Ether, nitrous oxide, phenobarbital, and pentobarbital produced a significant decrease in kidney NPSH. Thiamylal and chlorpromazine had no effect; whereas thiopental produced a slight increase. Cold had no effect on the kidney NPSH of animals given ether, nitrous oxide, phenobarbital and thiamylal. The thiopental and chlorpromazine groups exposed to cold showed a decrease in kidney NPSH.

Thiopental appeared to increase the blood NPSH (105 ± 7.1 μM% to 133 ± 4.8 μM%, $P = <.05$). No significant change in blood NPSH was observed when chlorpromazine hydrochloride was administered.

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TABLE I. Effect of Chlorpromazine and Anesthetics on Liver and Kidney Non-Protein Sulfhydryl Compounds in Normothermic and Hypothermic Rats.

Treatment	Body temp., °C	No. of animals	μM % NPSH in liver	Probability† With control	Probability† Hypothermic with normothermic	μM % NPSH in kidney	Probability With control	Probability Hypothermic with normothermic
Control	39	52	852 ± 7*			508 ± 6		
Chlorpromazine hydrochloride	20	9	567 ± 15		<.20	396 ± 17		<.01
	39	9	604 ± 21	<.01		499 ± 18	<.50	
Ether	18	10	693 ± 17		<.10	421 ± 17		>.50
	39	7	619 ± 28	,,		432 ± 28	<.05	
Nitrous oxide	18	8	579 ± 21		,,	406 ± 21		>.40
	39	6	488 ± 24	,,		404 ± 11	<.01	
Phenobarbital sodium	18	8	597 ± 24		<.01	500 ± 24		>.05
	39	8	705 ± 13	,,		450 ± 17	<.02	
Pentobarbital sodium	18	19	636 ± 12		>.10	401 ± 35		>.10
	39	20	655 ± 20	,,		341 ± 23	<.01	
Thiopental sodium	18	20	540 ± 15		<.01	415 ± 10		<.01
	39	18	658 ± 18	,,		551 ± 17	<.05	
Thiamylal sodium	18	8	582 ± 19		,,	512 ± 16		>.50
	39	8	679 ± 16	,,		516 ± 17	>.50	

* Stand. error of the mean. † P is probability derived from t values where $t = \frac{\text{Difference between means}}{\text{Stand. error of difference}}$.

Discussion. Hypothermia and stress depress metabolism in rats(5) and also decrease NPSH in actively metabolizing tissues(1,2). Since many anesthetic agents depress metabolism and produce a tendency toward hypothermia, it is not surprising that anesthetics decrease tissue NPSH. Adrenalin has been shown to lower the tissue NPSH(3). The sympatho-adrenal system is activated by most anesthetics(6), thus the lowered levels of the liver NPSH noted in these experiments may have been due to the activation of this system.

The usual effect of cold of lowering the liver NPSH was observed in the animals anesthetized with phenobarbital, thiopental, and thiamylal. However, it appears that chlorpromazine and pentobarbital may have paralyzed the mechanism activated by cold which decreases liver NPSH. Most anesthetics which caused a lowering of kidney NPSH also prevented any further diminution by exposure to cold. The only exceptions were chlorpromazine and thiopental. Since thiopental contains a sulfur atom, it is possible that the higher kidney levels in the normothermic category may represent concentrations of sulfhydryl groups from the anesthetic in the excretory process. Increased blood levels of NPSH were also observed when this drug was given. Since thiamylal was given at only one-third the level of thiopental, this slight effect would not be seen. The decrease in kidney NPSH in the hypothermic thiopental and chlorpromazine groups is essentially the same effect as that observed when control animals were exposed to cold and restraint(7).

Summary. 1. Female rats were divided into groups and given chlorpromazine or anesthetic agents. One-half of each group was exposed to cold. The total non-protein sulfhydryl concentration (NPSH) of liver and kidney was determined and compared with similar values for control animals. Chlorpromazine and all anesthetics produced a decrease in liver NPSH. Cold further decreased the NPSH of animals anesthetized with phenobarbital, thiamylal, and thiopental; however, cold had no effect on liver NPSH of the chlorpromazine and pentobarbital groups. 2. Ether, nitrous oxide, and phenobarbital de-

creased kidney NPSH. No change was observed in the chlorpromazine and thiamylal groups, but a slight increase in kidney NPSH occurred in the thiopental group. The possible relationship of anesthetics on tissue NPSH and metabolism is discussed.

1. Bartlett, R. G., and Register, U. D., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 708.
2. ———, *ibid.*, 1954, v86, 397.
3. Register, U. D., and Bartlett, R. G., Jr., *Science*, 1954, v120, 109.
4. Benesch, R. E., and Benesch, R., *Arch. Biochem.*, 1950, v28, 43.
5. Adolph, E. F., *Am. J. Physiol.*, 1950, v161, 359.
6. Salter, W. T., *A Textbook of Pharmacology*, W. B. Saunders Co., Philadelphia, 1952, p165,166.
7. Register, U. D., and Bartlett, R. G., Jr., *J. Biol. Chem.*, 1955, v212, 741.

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Effect of Administered Ascorbic Acid on Nitrogen Metabolic Response to Thermal Trauma. (22078)

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Numerous investigators have confirmed Cuthbertson's(1) observations of an increase in urinary nitrogen excretion by injured patients and animals. Lowered excretion of ascorbic acid, decreased adrenal and plasma ascorbic acid content, and decreased tissue ascorbic acid "saturation" as judged by load tests have also been observed during the period of increased urinary nitrogen excretion (2-5). These changes in ascorbic acid and nitrogen metabolism may be interrelated; ascorbic acid may be important in adrenal cortical function, and there is evidence to indicate that the nitrogen metabolic changes after injury are dependent, in part, on the level of adrenal activity. Ascorbic acid administered subcutaneously to normal rats was shown by Noble and Toby(6) to reduce urinary nitrogen excretion for at least 34 hours, the period of their observations. Further, rats traumatized by leg clamping and given ascorbic acid, either subcutaneously or orally, excreted less nitrogen in the first 34 hours after injury, the period of their observations, than rats similarly injured but not given ascorbic acid.

Accordingly, we have investigated the effects on nitrogen excretion of supplemental ascorbic acid given to rats with severe thermal injury throughout the period of negative nitrogen balance (2½ weeks following injury).

Methods. Thirteen male young adult albino rats of the Wistar strain were adapted over a period of 6 days to the force-feeding of a liquid diet(7,8). From the end of the adaptation period to the end of the experiment, each of the animals was given 10 cc of the diet 2 times daily. According to chemical analysis, each animal received 640 mg of nitrogen every 2 days. By calculation, this diet yielded about 0.2 calorie per gram of body weight per day and was made up, on a caloric basis, of 18% protein, 63% carbohydrate, and 19% fat. Six days after rats reached full feeding, they were placed in metabolism cages from which acidified urine and feces were collected every 2 days. Total nitrogen in urine, feces, and diet was determined by micro-Kjeldahl analysis(9). Urinary amino nitrogen was estimated by the colorimetric ninhydrin technic(10); no correction was made for urea which reacts with ninhydrin to the extent of 3%. Rats were weighed every other day. After a 12-day control period, all rats were lightly etherized and deep thermal injuries produced over 30-35% of their body surfaces by dipping their clipped backs into hot water (73°C) for 30 seconds

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TABLE I. Nitrogen Metabolism in Thermally Injured Rats: Effect of supplemental ascorbic acid.

Day of study	Body wt (g)		Urinary amino N (mg/48°)†		Urinary total N (mg/48°)†	
	Controls	Ascorbic acid supplemented	Controls	Ascorbic acid supplemented	Controls	Ascorbic acid supplemented
1- 2	295 ± 6*	278 ± 9	29.9 ± 2.1	30.7 ± 3.9	534 ± 48	504 ± 34
3- 4	286 ± 8	271 ± 8	32.5 ± 2.0	31.1 ± 4.1	515 ± 46	499 ± 43
5- 6	287 ± 8	274 ± 8	31.8 ± 1.7	29.3 ± 1.3	502 ± 24	491 ± 32
7- 8	293 ± 7	278 ± 7	29.7 ± 4.1	31.5 ± 4.1	500 ± 23	482 ± 48
9-10	293 ± 6	279 ± 8	(lost)	(lost)	513 ± 60	475 ± 28
11-12	296 ± 5	282 ± 7	31.6 ± 3.7	34.9 ± 2.6	506 ± 56	519 ± 52
13-14‡	296 ± 5	283 ± 7	37.5 ± 2.6	41.3 ± 10.8	700 ± 98	702 ± 102
15-16	307 ± 9	297 ± 13	34.0 ± 4.7	37.2 ± 6.2	704 ± 88	718 ± 106
17-18	298 ± 11	279 ± 10	40.8 ± 2.8	40.8 ± 2.1	741 ± 43	746 ± 48
19-20	287 ± 10	267 ± 7	39.3 ± 3.8	43.5 ± 5.0	698 ± 77	725 ± 74
21-22	282 ± 9	261 ± 9	37.1 ± 3.1	41.3 ± 4.1	678 ± 35	733 ± 81
23-24	276 ± 11	260 ± 9	39.3 ± 6.3	42.7 ± 6.9	633 ± 75	624 ± 91
25-26	272 ± 12	254 ± 11	44.3 ± 6.9	38.4 ± 7.2	716 ± 102	609 ± 101
27-28	270 ± 19	245 ± 22	41.0 ± 4.9	47.1 ± 6.5	697 ± 107	695 ± 58
29-30	260 ± 11	250 ± 14	35.4 ± 8.9	37.1 ± 5.9	603 ± 117	581 ± 32

* Stand. dev. † 48° intake, 640 mg N (constant). ‡ All rats thermally injured on day 13.

according to the method of McCarthy(11). All rats were maintained on the constant tube-fed diet. Seven of the 13 animals received supplemental ascorbic acid; the first dose (25 mg) was given intramuscularly 1½ hours after injury. Thereafter, 25 mg of ascorbic acid were given twice a day, orally, to each of these 7 rats. Ascorbic acid was added to their food just prior to each tube feeding. The study was conducted for 18 days after injury, by which time the nitrogen excretion in both groups had returned close to the pre-injury levels. At this time the injured skin of the back was in the form of a dried eschar; epithelization was slowly taking place at the periphery of the injured area.

Results. During the control period, all rats were in slight positive nitrogen balance. Their body weights were stable. The thermal injury was followed by a loss in body weight (after a transient gain due to edema in the injured area) and prompt and prolonged increase in urinary nitrogen excretion, total and amino. These changes were uninfluenced by the administration of ascorbic acid (Tables I and II; Fig. 1). (The data were analyzed

TABLE II. Nitrogen Metabolism in Thermally Injured Rats: Effect of supplemental ascorbic acid.

Day of study	Urinary + fecal total N (mg/48°)†		N balance (mg/100 g body wt/48°)†	
	Controls	Ascorbic acid supplemented	Controls	Ascorbic acid supplemented
1- 2	594 ± 45*	557 ± 40	+16 ± 15	+30 ± 14
3- 4	576 ± 35	551 ± 43	+23 ± 12	+33 ± 15
5- 6	552 ± 29	541 ± 38	+30 ± 10	+36 ± 13
7- 8	550 ± 16	547 ± 42	+31 ± 6	+33 ± 14
9-10	560 ± 26	524 ± 32	+27 ± 8	+42 ± 12
11-12	561 ± 50	568 ± 50	+27 ± 16	+26 ± 16
13-14‡	746 ± 108	749 ± 101	−36 ± 36	−39 ± 33
15-16	750 ± 93	766 ± 106	−36 ± 31	−42 ± 35
17-18	797 ± 47	811 ± 52	−53 ± 15	−61 ± 16
19-20	754 ± 105	789 ± 76	−40 ± 35	−56 ± 25
21-22	750 ± 25	799 ± 85	−39 ± 9	−61 ± 28
23-24	672 ± 67	690 ± 90	−12 ± 22	−19 ± 30
25-26	751 ± 98	669 ± 110	−41 ± 33	−11 ± 36
27-28	757 ± 103	761 ± 56	−43 ± 34	−49 ± 18
29-30	623 ± 95	636 ± 32	+ 7 ± 32	+ 2 ± 11

* Stand. dev. † 48° intake, 640 mg N (constant). ‡ All rats thermally injured on day 13.

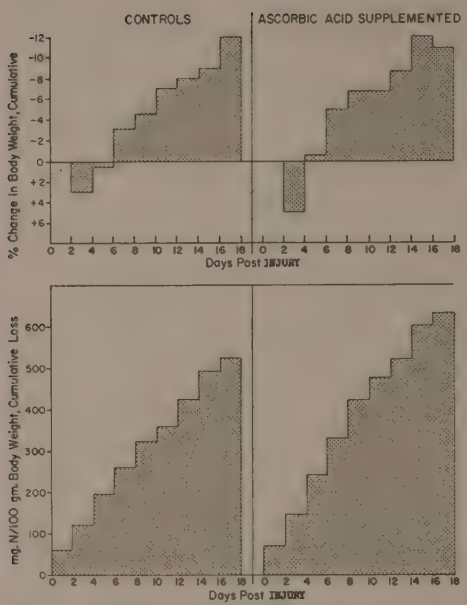

FIG. 1. Cumulative nitrogen balances and changes in body wt in thermally injured rats.

by the Fisher t test).

Prior to injury, the rats which were later to receive ascorbic acid were slightly lighter, about 5% on the average, than the controls. The rats in both groups lost about 12% of their pre-injury control weights during the 18-day period of increased urinary nitrogen excretion. About half of this weight loss can be accounted for by the observed increase in nitrogen excretion—if one assumes that the extra-urinary nitrogen was derived from tissue with approximately normal protein and water contents.

Lack of effect of ascorbic acid on the increase in urinary nitrogen excretion in this study is in apparent contradiction to the observation of Noble and Toby. However, the trauma employed in their study was leg crushing and their observations were limited to a brief (34-hour) period after injury; our observations were made in thermally injured rats and continued over a period of 18 days.

The rat, unlike man, does not require exogenous ascorbic acid for his daily normal requirements. Whether any supplemental ascorbic acid is needed by the rat following severe stress is not known. Ralli *et al.*(12) have recently studied the effect of the surgical removal of about 15% of the skin on the urinary ascorbic acid excretion by female rats. Ralli and her coworkers noted an increase in ascorbic acid excretion during the first 2 days after wounding, followed by a decrease to below the preoperative level for the next 2 weeks.

Conclusion. Loss of weight and increase in urinary nitrogen excretion, total and amino, by rats with severe thermal injury were unaffected by large doses of ascorbic acid begun shortly after injury and continued throughout the period of negative nitrogen balance (18 days). That these findings may be applicable to those species (man, monkey, and guinea pig) which depend on exogenous ascorbic acid for their normal requirements, unlike the rat, is suggested by the observations that marked increases in urinary nitrogen excretion following severe injury occur in patients receiving up to one g of vit. C daily(13).

1. Cuthbertson, D. P., *Biochem. J.*, 1930, v24, 1244.

2. Levenson, S. M., Green, R. W., Taylor, F. H. L., Robinson, P., Page, R. C., Johnson, R. E., and Lund, C. C., *Ann. Surg.*, 1946, v124, 840.

3. Lund, C. C., Levenson, S. M., Green, R. W., Paige, R. W., Robinson, P. E., Adams, M. A., MacDonald, A. H., Taylor, F. H. L., and Johnson, R. E., *Arch. Surg.*, 1947, v55, 557.

4. Andreae, W. A., Schenker, V., and Browne, J. S. L., *Fed. Proc.*, 1946, v5, 3.

5. Harkins, H. N., *Physiol. Rev.*, 1945, v25, 531.

6. Noble, R. L., and Toby, C. G., *J. Endocrinol.*, 1948, v5, 303.

7. Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 44.

8. Braasch, J. W., Bell, J. H., and Levenson, S. M., *Surgery*, 1950, v27, 743.

9. Ma, T. S., and Zuazaga, G., *Indust. and Engin. Chem.* (Analytical Edition), 1942, v14, 280.

10. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1948, v176, 337.

11. McCarthy, M. D., *J. Lab. Clin. Med.*, 1945, v30, 1027.

12. Ralli, E. P., Dumm, M. E., Kuhl, W. J., Gershberg, H., and Beck, E. M., *Am. J. Physiol.*, 1954, v179, 319.

13. Levenson, S. M., Lounds, E. A., and Morris, R., *Ann. N. Y. Acad. Sci.*, 1952, v56, 1.

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Site of Action of Magnesium in Treatment of Some Toxic Effects Produced by TEPP.* (22079)

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Major pharmacological actions of magnesium have been discussed in recent reviews, Engbaek(1); Smith, Winkler and Hoff(2). Various peripheral and central effects of the ion have been described, but, as noted by Engbaek(1), the question of relative sensitivities of central and peripheral structures to magnesium has not been settled.

It has been reported, McNamara, Koelle and Gilman(3), that magnesium sulfate, when used adjunctively with pentobarbital and atropine, is of value in treating poisoning produced by diisopropylfluorophosphate (DFP). The current investigation is an attempt to elucidate the site of action of magnesium in the treatment of certain effects produced by tetraethylpyrophosphate (TEPP).

Methods. Three types of experiments were performed. 1) Cats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), atropinized (1 mg/kg, i.m.) and respired artificially. Sciatic and femoral nerves of one leg were sectioned, and identical bi-polar platinum electrodes were inserted into the gastrocnemius muscles of this leg and of the opposite intact leg. Electrical activity of these muscles was recorded with a Grass electroencephalograph. In some of these experiments, electroencephalograms were recorded from screw electrodes placed in the skull. Recordings were made immediately prior to, and periodically after, poisoning with tetraethylpyrophosphate (TEPP) by injection of 0.3 to 2.0 mg/kg into the antibrachial vein. When skeletal muscle fasciculations had been produced by TEPP, magnesium sulfate or magnesium chloride (30-100 mg/kg in 5% solution) was intravenously. In a few experiments, after fasciculation had been abolished by the magnesium salt, the threshold for indirect stimulation of the gastrocnemius muscle of the intact leg was compared with that at the beginning of the experiments. 2. The sciatic nerve-gastrocnemius muscle technic of Wolff and Cattell (4) was used to study the response of muscle to supramaximal excitation of its motor nerve following intravenous doses of 30-130 mg/kg of magnesium sulfate in cats. 3) Rabbits were paralyzed by Intocostrin (1-2 units/kg i.v.) and were respired artificially. Dibutoline (0.5 mg/kg, i.m.) was administered because it is the authors' experience that this drug minimizes bronchial and cardiovascular effects of TEPP without preventing appearance of the "grand mal" type of electroencephalogram produced by the latter com-

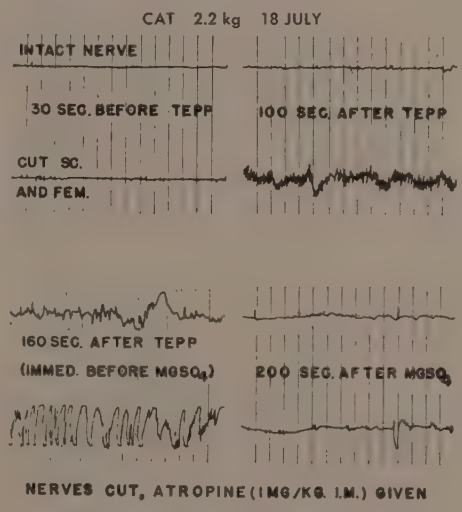

FIG. 1. Effect of magnesium sulfate (30 mg/kg i.v.) on fasciculation induced by TEPP (1 mg/kg i.v.) in cat gastrocnemius with intact innervation (upper traces) and with denervation approximately 2 hr before experiment (lower traces). Cat received atropine (1 mg/kg i.v.) about 2 hr before experiment, and given artificial respiration as necessary after poisoning. Tracings shown are similar to those obtained from 9 other cats treated similarly.

* Abstract of this work appears in *Fed. Proc.*, 1955, v14, 373.

TABLE I. Effect of Magnesium on Response of Gastrocnemius Muscle to Supramaximal Electrical Stimulation of Its Motor Nerve.

Cat No.	Dose $MgSO_4$ (mg/kg, i.v.)	Effect on contraction height
1	30	No significant effect
	100 (Immed.)*	About 70% reduction
2	30	No significant effect
	100 (immed.)	Complete block
3	50	No significant effect
	50 (Immed.)	About 85% reduction
4	50	No significant effect
	50 (Immed.)	About 50% reduction
5	50	No significant effect
	50 (Immed.)	Complete block
6	60	No significant effect
7	60	About 30% reduction
8	120	" 85% "

* Immed. = Immediately after 1st dose.

pound. A screw electrode was placed in the right frontal area of the skull, potential differences between this point and an indifferent skin area being recorded. TEPP was then administered. When increased electrical activity of the brain resulted, magnesium sulfate (30-150 mg/kg, i.v.) was administered in an effort to counteract the central effects of TEPP. Atropine (1-3 mg/kg, i.v.) was injected thereafter.

Results. Group I. Fig. 1 shows clearly that the site of action of magnesium in counteracting these fasciculations is peripheral because magnesium sulfate, in a dose of 30 mg/kg i.v., quickly abolished the fasciculatory response in both the decentralized leg and the leg with intact nerves. In other experiments, doses of up to 100 mg/kg of magnesium salt were needed to abolish fasciculation. In 4 of 5 experiments, it was noted that the threshold for indirect stimulation of the muscle following abolition of fasciculation by low doses of magnesium was similar to that of the prepoisoning control.

Group II. Table I illustrates the effect of magnesium sulfate on the response of the gastrocnemius muscle to supramaximal electrical stimulation of the sciatic nerve. Doses of magnesium (30 mg/kg, i.v.) which frequently abolished fasciculations in other experiments did not alter significantly the response of the muscle to motor nerve excitation. Large doses of the magnesium ion, however, reduced the muscles' contraction height.

Group III. Magnesium sulfate in doses as high as 150 mg/kg, i.v., did not abolish the abnormal brain activity in any of 6 rabbits. When atropine sulfate was then given (1-3 mg/kg, i.v.), the "grand mal" pattern was quickly abolished. Fig. 2 illustrates these results.

Discussion. The results of this investigation show that the site of action of magnesium sulfate in the treatment of some effects of TEPP intoxication is primarily peripheral. The magnesium salt was effective in abolishing fasciculations in both the leg with sectioned nerves and in that with intact innervation. The exact mechanism of this action has not been elucidated by the current experiments, but evidence has been obtained that the magnesium effect does not involve necessarily depression of neuromuscular transmission or of repetitive cerebral or spinal cord activity. The action probably is localised, therefore, to the skeletal muscle fiber.

It has been reported that magnesium reduces the output of acetylcholine from cholinergic nerve endings, Hutter and Kostial(5). Others, Del Castillo and Engbaek(6), have demonstrated that, although the main effect of magnesium on neuromuscular function is a diminution of the amount of acetylcholine released by the motor nerve endings, the magnesium ion raises the electrical threshold of the muscle membrane and also has a small depressant effect on the acetylcholine sensitivity of the end-plates. Both of these mechanisms may well play a part in the effect on the muscle fiber made to fasciculate by TEPP.

Summary. 1. The primary site of action of magnesium in the treatment of skeletal muscle fasciculations produced during TEPP intoxication is peripheral. 2. Magnesuim apparently has little, if any, value in the treatment of the central effects produced by this poison.

1. Engbaek, L., *Pharmacol. Rev.*, 1952, v4, 396.

2. Smith, P. K., Winkler, A. W., and Hoff, H. E., *Anesthesiology*, 1942, v3, 323.

3. McNamara, B. P., Koelle, G. B., and Gilman,

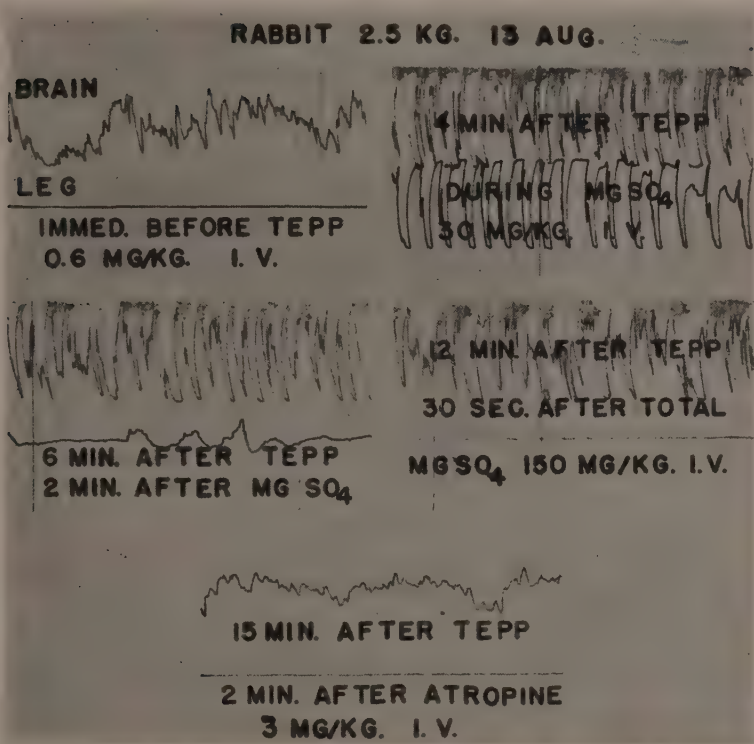

FIG. 2. Comparative effectiveness of magnesium sulfate (30 mg/kg i.v. and 7.5 min. later 120 mg/kg i.v.) on EEG hyperactivity (upper traces) and on fasciculation of gastrocnemius (lower traces) induced by TEPP (0.6 mg/kg i.v.) and comparative effectiveness of magnesium sulfate and of atropine sulfate (3 mg/kg) on EEG hyperactivity (last two panels). Rabbit received Dibutoline (0.5 mg/kg i.m.) and Intocostrin (2 units/kg i.v.) before start of experiment, and respired artificially throughout experiment.

A., *J. Pharmacol. Exp. Therap.*, 1946, v88, 27.

4. Wolff, H. G., and Cattell, McK., *Arch. Neurol. Psychiat.*, 1934, v32, 81.

5. Hutter, O. F., and Kostial, K., *J. Physiol.*, 1953, v120, 53.

6. Del Castillo, J., and Engbaek, L., *J. Physiol.*, 1953, v120, 54.

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Presence of ACTH in Pituitary Gland of Pacific Salmon (*O. keta*). (22080)

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The existence of a relationship between the pituitary gland and interrenal tissue of fish (*Astyanax mexicanus*) has been reported by Rasquin(1). Following injections of ACTH of mammalian origin, the anterior interrenal tissue, which is considered homologous with the mammalian adrenal cortex, responds by hypertrophy, hyperplasia, and increased vascularization. Hatay(2-4), and Fontaine and Hatay(5-7) show that the corresponding tissue in the eel (*Anguilla anguilla* L) is rich in ascorbic acid and that following hypophysectomy, this component is decreased and the interrenal tissue has become atrophic. In contrast, Pickford(8) was unable to detect changes in the cortical tissue of *Fundulus* following hypophysectomy.

It seemed of interest to determine if a corticotropic substance exists in the teleost pituitary, which like mammalian ACTH, can deplete adrenal ascorbic acid in the hypophysectomized rat. Assays of lyophilized pituitary glands* and of certain pituitary fractions of a species of Pacific salmon (*Oncorhynchus keta*), which will be reported, show clearly the presence of ascorbic acid-depleting activity.

Materials and methods. Preparation of extracts. Pituitary glands from freshly caught salmon were preserved in solid carbon dioxide within 3 hours of sacrifice. Following lyophilization, the dried tissue was ground with mortar and pestle. Immediately before use a weighed amount of tissue was extracted in an appropriate volume of N/1000 hydrochloric acid. This preparation was injected without further processing. *Preparation of fractions.* Fraction #1. Lyophilized pituitary tissue was extracted at room temperature for 24 hours in a solution of glacial acetic acid and acetone;† one g of tissue in 16 ml of acid and 2.5 ml acetone. One volume of distilled water was then added. After thorough mixing the suspension was centrifuged, the insolubles being preserved by lyophilization for further study. To the supernatant acetone was added, with stirring, until heavy turbidity was evident. At this point additional acetone was added cautiously until a flocculent precipitate formed, leaving a clear supernatant. The solids were collected by centrifuging, and washed twice with ethyl ether. The washed precipitate was redissolved in water, enough glacial acetic acid being added to assure solution. After warming at 37°C for a few minutes to remove excess ether, the solution was frozen and lyophilized. Yield: approximately 13% of starting weight. Fraction #2. To the supernatant fluid remaining after the precipitation of Fraction #1 was added at least one volume of acetone. If necessary,

TABLE I. Adrenal Ascorbic Acid Depletion in Hypophysectomized Rats following the Intravenous Administration of Various Pituitary Preparations of the Pacific Salmon (*O. keta*).

Preparation used	Dose* (μg)	No. of animals	Adrenal ascorbic acid depletion (mg %)
Lyophilized tissue	25	3	73, 68, 105
	100	3	101, 177, 119
Fraction #1	50	2	222, 188
	100	3	214, 167, 182
Fraction #2	50	3	137, 105, 150
	100	3	149, 156, 70
Fraction #1	10	3	83, 138, 97
	20	2	124, 96
	40	4	176, 143, 195, 162
Fraction #2	12.5	3	37, 79, 62
	25.0	3	72, 64, 84
	50.0	3	159, 134, 177

* Dose based on 100 g of body wt. Left adrenals removed immediately before intravenous administration of test dose. Right adrenals removed 1 hr following test dose.

* Pituitary glands were kindly supplied by Mr. Roger Burrows and his colleagues of the Fish and Wildlife Service, U. S. Department of Interior, Entiat, Wash.

† This procedure is a modification of the method reported by Payne, Raben and Astwood(9) for extraction of hog pituitary glands.

TABLE II. Adrenal Ascorbic Acid Depletion Assays in Hypophysectomized Rats Injected with Pituitary Preparations of the Pacific Salmon *(O. keta)* and with U.S.P. Standard Corticotropin.

No. of animals	Test material	Test dose per 100 g body wt	Mean response, adrenal ascorbic acid depletion	Assay data
8	LSP*	25 μg	110	$S_c = \pm 20$†
8	"	50 "	131	$b_c = 86.7$
8	"	100 "	160	$\lambda_c = .23$
7	USP Std	.5 mU§	103	$R \frac{(USP)}{(LSP)} = 48.9$‡
7	"	1.0 "	134	95% limits 35.7-66.8
7	"	2.0 "	158	
3	Fraction #1	25 μg	109	$S_c = \pm 32$
4	"	50 "	168	$b_c = 143$
3	USP Std	.5 mU	93	$\lambda_c = .22$
3	"	1.0 "	120	$R \frac{(USP)}{(Fr.1)} = 29.4$
3	Fraction #2	25 μg	85	
3	"	50 "	95	$S_c = \pm 24$
3	"	100 "	130	$b_c = 84.3$
3	USP Std	.5 mU	93	$\lambda_c = .29$
3	"	1.0 "	120	$R \frac{(USP)}{(Fr.2)} = 52.8$
3	"	2.0 "	149	

Assays conducted according to the method of Sayers *et al.* Left adrenals removed immediately before injection of test dose. Right adrenals removed 1 hr following test dose.

* LSP = Lyophilized salmon pituitary tissue.

† S = Stand. dev.; b = slope; λ = s/b; subscript 'c' for combined data.

‡ R = Ratio of potency.

§ mU = Milliunits.

further acetone was added to reduce the turbid suspension to a flocculent precipitate. The solids were collected by centrifuging and prepared for lyophilization as with Fraction #1. Yield: approximately 11% of starting weight. *Assays.* Adrenal ascorbic acid depletion assays were conducted according to the method of Sayers, Sayers, and Woodbury (10). Male Sprague-Dawley rats, 125-150 g body weight, were hypophysectomized 24 hours prior to assay. Test preparations dissolved in N/1000 hydrochloric acid, were injected, after laparotomy, via the vena cava. Preliminary tests were made to determine whether ACTH activity was present. The results of these were used to estimate dose levels in assays against the USP standard corticotropin.

Results. Data obtained in preliminary tests are presented in Table I. It is apparent that, in these preparations from salmon pituitary tissue, a physiological activity is present which is qualitatively the same as that observed in pituitary preparations of mammalian origin. Potency assays against the USP standard corticotropin are summarized in Table II. The results indicate an ACTH content in the lyophilized tissue of about 20 milliunits per mg. Because of difficulties in field conditions under which these glands were collected, freezing in solid carbon dioxide was delayed 2 to 3 hours after removal. This lapse of time may have resulted in loss of physiological activity. Hence it is possible that freshly frozen glands would show a greater ACTH content.

Assay data on Fractions #1 and #2 indicate some concentration of ACTH activity in the former. On a weight basis Fraction #2 appears about as active as the starting material.

Discussion. The findings reported establish the presence of ACTH activity in the teleost pituitary. A recent report by Nelson, O'Connell, and Haines(11) describes the presence of ACTH activity in sardine fish meal. The results described here may account

in part for this finding, if small quantities of pituitary tissue were present in the meal.

The presence in the teleost of a pituitary component capable of producing adrenal cortical stimulation in the mammal suggests that the protein or peptide structure responsible for this activity was of metabolic importance early in the evolutionary development of the species. Whether or not the role of ACTH in the phenomena associated with response to stress in mammals is the same in fish remains to be determined.

Summary. Lyophilized pituitary tissue of the Pacific salmon (*O. keta*) and pituitary fractions, were examined for ACTH activity in hypophysectomized rats. The results indicate the presence of a component in the teleost pituitary gland capable of depleting the stores of adrenal ascorbic acid in the rat. In this respect the preparations are qualitatively identical with corticotropins (ACTH) of mammalian origin.

The authors wish to acknowledge with gratitude the interest in this work of Dr. O. H. Robertson, Stanford University, who first introduced them to the endocrine problem in fish.

1. Rasquin, P., *J. Exp. Zool.*, 1951, v117, 317.
2. Hatey, J., *C. R. Soc. Biol.*, 1952, v146, 566.
3. ———, *ibid.*, 1954, v148, 324.
4. ———, *ibid.*, 1954, v148, 231.
5. Fontaine, M., and Hatey, J., *ibid.*, 1953, v147, 217.
6. ———, *Bull. Inst. Oceanogr. Monaco*, 1954, v1037, 7.
7. ———, *J. Physiol.* (Paris), 1954, v46, 364.
8. Pickford, G. E., *Bull. Bingham Oceanogr. Collection*, 1953, v14, 5.
9. Payne, R. W., Raben, M. S., and Astwood, E. B., *J. Biol. Chem.*, 1950, v187, 719.
10. Sayers, M., Sayers, G., and Woodbury, L., *Endocrinology*, 1948, v42, 379.
11. Nelson, J. W., O'Connell, P. W., and Haines, W. J., *Science*, 1954, v119, 379.

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Rate of Glycogenesis in the Liver of the Rat After Parenteral Administration of Glucose, Fructose and Invert Sugar.* (22081)

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It is generally considered that fructose forms glycogen in the liver more rapidly than glucose. This assumption is based upon *in vitro* experiments(1) and studies in which the two sugars were given to rats by mouth (2). Since in clinical practice sugars are administered intravenously for supplying calories to patients unable to take food by mouth, it was considered of interest to study the rate of glycogenesis in the liver when glucose, fructose, and invert sugar are given parenterally.

Method. Normal white rats, weighing 200 to 300 g, were fasted for exactly 24 hours, water being allowed *ad lib.* Sugar, in 10% solution, was injected intraperitoneally. A dosage of 2 g per kg of body weight was used. This dosage was selected because it was found by experiment to give a suitable load to the animal and it is below the level at which utilization might be diminished by biological limitations. A carefully measured quantity of sugar solution was injected intraperitoneally into the fasted rat. At different intervals after administration, animals were anaesthetized with nembutal and the whole liver was removed for analysis. Liver glycogen was determined by an anthrone method developed in our laboratory(3). Our procedure was to give sugar solution to 6 rats and the same volume of physiological saline to 2 control animals. Liver glycogen determinations were then carried out upon the group of 8 animals. Groups of animals were sacrificed at approximately hourly intervals for periods.

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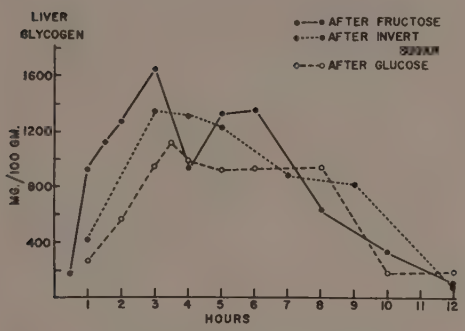

FIG. 1. Rate of glycogenesis in liver in fasted rats after intraperitoneal injection of fructose, invert sugar and glucose. Dosage was 2 g/kg of body wt.

extending up to 12 hours after administration. Observations were made following the administration of glucose, fructose and invert sugar. Comparative results were thus obtained for the 3 sugars using identical experimental procedures.

Results are shown in Fig. 1. Liver glycogen formation after fructose injection was approximately twice as fast as after glucose injection for 3 hours; at the fourth hour the curve after fructose administration dropped sharply but returned to a higher level than the glucose and invert sugar curves at the 5 and 6-hour points and then sloped off at about the same level as for the other two sugars between 7 and 12 hours after administration. The curve showing liver glycogen content after glucose is the lowest level observed for the 3 sugars. The curve showing liver glycogen values after invert sugar injection has a level about midway between the curves for fructose and glucose for 6 hours after administration and then occupies a level about the same as the other 2 sugars until the twelfth hour. The liver glycogen content 12 hours after administration returned to the pre-injection level for each sugar.

At the highest point of the glycogen curve for each sugar the per cent of the administered dose that had been converted into glycogen was 28.8 for fructose, 20.3 for invert sugar and 18.1 for glucose.

The intermediate position of the invert sugar curve is as would be expected of this sugar, since it is an equimolar mixture of glucose and fructose. The sharp dip in the curve after fructose at the 4-hour period is difficult to explain. The blood glucose after fructose injection had reached its highest level, 120 mg%, at the 5-hour point. Since fructose gives rise to increased blood glucose, possibly the effect of the latter caused the upturn of the glycogen curve at this point after fructose administration. This suggestion is supported by the fact that the curve after invert sugar maintained a steady level at the 4-hour point which shows the influence of glucose at this time.

Summary. Rate of glycogenesis in the liver of the rat after intraperitoneal injection of sugar solution was found to be highest after fructose, lowest after glucose, and intermediate between fructose and glucose after invert sugar. During the first 3 hours after injection the rate of glycogen formation was approximately twice as fast after fructose as after glucose, a finding that explains, in part, the more rapid disappearance from the blood of fructose than glucose after parenteral administration.

1. Vestling, C. S., Mylorie, A. K., Irish, U., and Grant, N. H., *J. Biol. Chem.*, 1950, v185, 789.

2. Cori, C. F., *ibid.*, 1926, v70, 577.

3. Carroll, N. V., Longley, R. W., and Roe, J. H., Abst., 127th Meet., Am. Chem. Soc., 1955, 23C.

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Implantation of Functional Erythropoietic Elements Following Total-Body Irradiation.* (22082)

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(Introduced by A. Hollaender.)

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It has been demonstrated that recovery from lethal doses of X irradiation is promoted by the injection of living, nonirradiated bone marrow(1). It has been postulated that the introduced marrow cells proliferate and are responsible for recovery by cellular repopulation, or that they elaborate some humoral substance which stimulates recovery of the indigenous marrow. Transitory activity of the injected cells pending recovery of the irradiated marrow has also been suggested as the beneficial role played by the transfused marrow. It seems reasonable that marrow cells injected into an irradiated animal might proliferate and contribute cellular elements to the peripheral circulation of the host because of (a) the inhibiting effects of total-body irradiation on the immune response (2,3), (b) the demonstration of homologous transplantation into embyronic hosts(4-6) and of the postpartum tolerance by these hosts of skin transplants from the donor (7-10), and (c) the acquired tolerance by irradiated marrow-injected mice of skin grafts from the marrow donor(11). Demonstration of the establishment of a functional implant of foreign bone marrow in an irradiated animal would indicate that the amount of genetic difference which can be tolerated between the donor of a tissue transplant and the recipient may be increased by irradiation of the recipient, and would lend support to the cellular repopulation hypothesis as a factor in radiation recovery. Experiments with rats are described, in which marrow carrying an immunogenetic marker is utilized. It is shown that implanted marrow cells do contribute erythrocytes to the peripheral blood of irradiated animals for extended periods of time.

Experimental. Cellular antigens C and D in the rat are controlled by a single pair of alleles; erythrocytes of the 2 homozygotes, C/C and D/D, carry antigens C and D respectively, and those of the heterozygote, C/D, carry both antigens C and D(12). Cells of either homozygous type may be detected in a mixture of both by the specific agglutination of each with its homologous antiserum. Animals segregating for these genes as well as specific reagents against each antigen were kindly supplied by Dr. Ray D. Owen of the California Institute of Technology. Rats of one antigenic type were irradiated and subsequently injected with bone marrow of another type; the irradiation and injection schedules used in these experiments are described in Table I. Marrow was forced from the femora of freshly decapitated rats, suspended in Tyrode's solution, and immediately injected into the jugular vein of the anesthetized recipient. In the first experiment, the marrow from one femur was injected into each recipient, whereas marrow from two femora was generally used in Experiments 2 and 3. Unirradiated controls were similarly injected with the carrier. At various intervals following administration of the bone marrow, a drop of blood was collected from the tail of each surviving marrow-injected animal and suspended in citrate.

TABLE I. Schedule of Treatment of Rats Injected with Immunogenetically-Labeled Marrow.

Exp. No.	No. of animals	Dose (r)	Type of host	Type of implant	Time of marrow inj. post-irrad. (hr)
1a	10	900	C or D	CD	0– 3
b	10	900	CD	—	—
c	10	0	C or D	CD	0– 3
2a	11	750	C or D	CD	18–24
b	7	750	CD	—	—
3a	21	750	C or D	D or C	18–24
b	11	750	CD	—	—
c	7	0	C or D	D or C	18–24

* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

TABLE II. Results of Bone Marrow Administration following Total-Body Irradiation. Each day's results are represented by a fraction; numerator represents No. of animals showing in peripheral blood erythrocytes of implanted antigenic type, and denominator represents No. of surviving animals; on days when no blood typing was done, no numerator is present.

		Time after irradiation (days)										
Exp. No.	Treatment	0	6	8	10	14	18	20	22	28	35	46
1 a	X-ray + marrow	–/10	0/ 5	–/ 3	–/ 3	1/ 1	1/ 1	–/ 0				
b	X-ray + Tyrode's	–/10	–/ 8	–/ 0								
c	Marrow	–/10	–/10	–/10	–/10	0/10						
2 a	X-ray + marrow	–/11	–/11	0/ 4	–/ 4	3/ 3	3/ 3	3/ 3	2/ 2	2/ 2	2/ 2	1/1
b	X-ray + Tyrode's	–/ 7	–/ 7	–/ 6	–/ 6	–/ 5	–/ 5	–/ 5	–/ 4	–/ 4	–/ 3	–/3
3 a	X-ray + marrow	–/21	–/21	–/19	0/17	11/16	–/16	12/14	–/14	12/14	12/14	
b	X-ray + Tyrode's	–/11	–/11	–/10	–/ 8	–/ 5	–/ 5	–/ 5	–/ 4	–/ 4	–/ 4	
c	Marrow	–/ 7	–/ 7	–/ 7	0/ 7	0/ 7	–/ 7	0/ 7	–/ 7	0/ 7	0/ 7	

Each sample was washed once in 100 volumes of saline and diluted to a 2% cell suspension; this cell suspension was tested against anti-implant-type serum, against saline, and in some instances against antihost-type serum. Antisera were used in concentrations sufficient to effect complete agglutination of a 2% suspension of homologous erythrocytes. The mixtures were lightly centrifuged and examined for evidence of agglutination upon resuspension of the sedimented cells. The resuspended mixture was often examined microscopically to detect cell aggregates too small to discern with the unaided eye.

The *results* of the experiments are summarized in Table II. By the end of the second week, erythrocytes from the implant could be demonstrated circulating in the peripheral blood of most of the surviving irradiated, marrow-injected animals; the injected but unirradiated animals, on the other hand, showed no evidence of implanted cells at any time. The first indications of erythrocytes from the implant were generally visible only microscopically. A positive microscopic agglutination was usually followed within a few days by positive macroscopic agglutination. The length of time a marrow implant will persist is unknown; at the time of this communication, one mosaic animal from Exp. 2 is still living after 147 days, and 12 animals from Exp. 3 are still alive after 95 days with no evidence of implant regression.

The methods described by Wilkie and Becker(13) for quantitative determination of hemagglutinin titers were modified slightly and used to estimate the composition of the erythrocyte populations of the surviving rats in these experiments. First estimates on the thirty-seventh day postirradiation on 3 animals from Exp. 3 showed about 85% implant- and 15% host-type cells in two animals, and less than 5% implant- and 90% host-type cells in one animal. On the 65th and 95th days, postirradiation values for 12 animals ranged from less than 10% implant-type cells to close to 90%, the majority of the animals showing more than 60% implant-type cells. The sum of independent estimates of the percentage of host-type and implant-type cells was close to 100%, and agreement between estimates made on the 37th, 65th and 95th days was excellent. The sole survivor of Exp. 2 showed about 95% implant-type and 5% host-type cells on days 91, 112 and 147 postirradiation.

Discussion. The factors which determine the fate of tissue transplants have long been of great interest. It is known that the compatibility of a transplant is a function of the genetic similarity between the donor and the recipient; transplantation between identical twins or between individuals of a highly inbred strain is usually successful, whereas transplantation between less closely related individuals of the same species or between species is generally unsuccessful. There are, however, conditions under which successful transplantation can occur in the presence of greater genetic diversity between recipient and donor. If an embryonic animal is confronted with a foreign antigen throughout the development of its antibody-producing system, the postpartum reactions of the animal to the same antigen are profoundly affected. If the antigen is cellular, the foreign cells

may continue to divide and may actually differentiate and function in the host animal; this has been demonstrated for naturally administered erythroid elements in dizygotic twins in cattle(4,5) and in man(6) and for artificially administered erythroid elements in the rat [Ripley and Owen in(14)]. In all cases, the administered cells become established and produce mature erythrocytes giving rise to persistent erythrocyte mosaicism. Anderson *et al.*(10) have shown in cattle that mosaicism of one animal for erythrocytes of his twin renders that animal tolerant of skin grafts from the twin. Billingham *et al.*(8,9) have further shown that mice and chickens injected as embryos with homologous cells are more tolerant as adults of skin grafts from the donor of the injected cells.

It is probable that the reaction of an irradiated rat to a foreign cellular antigen is analogous to the reaction of an embryonic animal to the same cellular antigen. It has been shown by Dixon *et al.*(2) that the immune response of the rabbit can be temporarily eliminated by total-body irradiation in the dose range used in the experiments reported in this communication. It might be postulated that postirradiation administration of the cellular antigen results in continuous exposure of the reforming antibody-producing system to the foreign antigen; consequently, the reformed system is tolerant of a foreign antigen to which the original was not tolerant. Indeed, Main and Prehn(11) have shown that irradiated mice of one strain injected with bone marrow of a second strain are subsequently tolerant of skin grafts from the second strain; this observation, they conclude, is consistent with the hypothesis that the injected marrow elements proliferate.

Demonstration of persistent erythrocyte mosaicism in which (in some cases) more than 80% of the erythrocytes in the peripheral circulation have been derived from the implanted marrow makes it unnecessary to postulate a humoral factor or a transitory activity of the implant as the mechanism responsible for recovery of the erythropoietic and inferentially myelopoietic potential in irradiated rats treated with homologous marrow. The possibility remains that the introduced cells may produce, in irradiated animals, some substance which stimulates recovery and proliferation of marrow. It is recognized that an important contribution of erythropoietic recovery to general radiation recovery is not demonstrated in the data reported here, since no recovery-promoting effects of the marrow are evident (Table II). This observation is consistent with what is known of radiation death in rats, where the contribution of intestinal damage outweighs and precedes that of marrow damage(15).

An explanation tentatively hypothesized by Cole(16) to explain the radiation recovery brought about by the administration of living cells is that the injected cells repair genetic damage incurred by the irradiated cells by a process similar to bacterial transformation or transduction; this hypothesis is to explain his finding that nuclei and deoxyribonucleic acid extracts of spleen cells have recovery-promoting activity. On this hypothesis, it might be suggested that in the present case the cells of the implant antigenic type are descendants of a selected subpopulation of irradiated cells in which the damage has been repaired by transformation. Bacterial transformation occurs independently for unlinked loci(17,18). Complete transformation of a diploid cell from one homozygous type to another would consequently involve the intermediate formation of the heterozygous type, *i.e.*, CC→CD →DD, which was not detected. That the estimated proportions of CC and DD cells account for all erythrocytes is clearly incompatible with the concept of transformation at the chromosomal level.

Summary. Rats have been irradiated with doses of X-rays in the LD_{50} range and subsequently injected with homologous bone marrow carrying an immunogenetic marker. Erythrocytes carrying the label can be detected in the peripheral circulation of these animals within 2 to 3 weeks after administration of the marrow; unirradiated but marrow-injected controls do not show erythrocytes from the implant. Marrow implants have persisted and functioned for as long as 147 days at the time of this communication.

The implanted marrow contributes as much as 80% of the peripheral erythrocytes.

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1. Lorenz, E., and Congdon, C. C., *Ann. Rev. Med.*, 1954, v5, 323.
2. Dixon, F. J., Talmage, D. W., and Mauer, P. H., *J. Immunol.*, 1952, v68, 693.
3. Taliaferro, W. H., and Taliaferro, L. G., *ibid.*, 1951, v66, 181.
4. Owen, R. D., *Science*, 1945, v102, 400.
5. Owen, R. D., Davis, H. P., and Morgan, R. F., *J. Heredity*, 1946, v37, 290.
6. Dunsford, I., Bowley, C. C., Hutchinson, A. M., Thompson, J. S., Sager, R., and Race, R. R., *Brit. Med. J.*, 1953, v2, 81.
7. Billingham, R. E., Lampkin, G. H., Medawar, P. B., and Williams, H. L., *Heredity*, 1951, v6, 201.
8. Billingham, R. E., Brent, L., and Medawar, P. B., *Nature*, 1953, v172, 603.
9. ———, *Ann. N. Y. Acad. Sci.*, 1955, v59, 409.
10. Anderson, D., Billingham, R. E., Lampkin, G. H., and Medawar, P. B., *Heredity*, 1951, v5, 379.
11. Main, J. M., and Prehn, R. T., *J. Nat. Cancer Inst.*, 1955, v15, 1023.
12. Owen, R. D., *Genetics*, 1948, v33, 623.
13. Wilkie, M. H., and Becker, E. L., *J. Immunol.*, 1955, v74, 192.
14. Horowitz, N. H., and Owen, R. D., *Ann. Rev Physiol.*, 1954, v16, 81.
15. Swift, M. N., Taketa, S. T., and Bond, V. P., *Radiation Research*, 1954, v1, 241.
16. Cole, L. J., and Ellis, M. E., *ibid.*, 1954, v1, 347.
17. Hotchkiss, R. D., *J. Cellular Comp. Physiol*, 1955, v45, Suppl. 2, 1.
18. Lennox, E. S., *Virology*, 1955, v1, 190.

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A Simple Circulatory Bypass System.* (22083)

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Some method of total circulatory bypass is necessary to accomplish open intracardiac surgery. Ideally such a system should entail a minimum of danger to the subjects accompanied by a maximum of simplicity of working parts and operability. The use of homologous and heterologous lungs in combination with some type of mechanical pump has been shown by others(1,2) to most closely approach this ideal. This is a report of experimental work with an homologous lung system which has been further simplified.

Methods. Mongrel donor dogs weighing 10 to 20 kg were bled to death after receiving one mg of heparin per kilo of body weight. Heparin was then added to this blood in amounts of one mg per 100 cc of blood. This blood was used to prime the extracorporeal system, to replace blood loss, and to maintain the operated dog's blood pressure during surgery. No blood was given to any of the dogs after completion of the experimental surgical procedure. Following exsanguination of the donor dog, the left lung was removed and the bronchus and pulmonary artery were cannulated, the former being connected to a respirator and the latter to the discharge end of a 300 cc graduated cylinder. Experimental dogs weighing from 9 to 20 kg were then anesthetized with morphine and nembutal and the

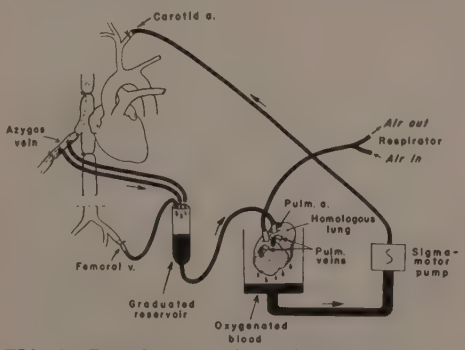

FIG. 1. Cannula connections of homologous lung blood oxygenator.

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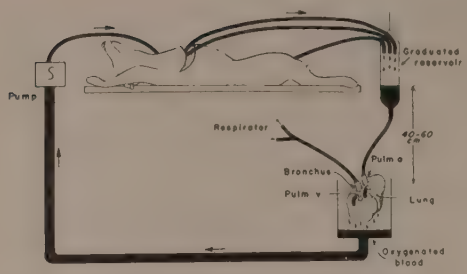

FIG. 2. General arrangement of homologous lung blood oxygenator.

femoral artery and vein cannulated and connected to an electronic recording manometer. Cannulae were also placed in the other femoral vein, the distal portion of the azygos vein, and the superior vena cava via the proximal portion of the azygos vein. These cannulae were all led to the intake portion of the 300 cc graduated reservoir. Another cannula was placed in the proximal portion of the dog's carotid artery and led through a Sigmamotor pump to a bottle collecting the oxygenated blood. All cannulae were filled with a saline solution containing one mg of heparin per kilo of body weight of the dog. The details of the various cannula connections are shown in Fig. 1 and the general arrangement of the lung and pump is shown in Fig. 2. During operation of the extracorporeal system, venous blood is drained into the graduated cylinder, which can be used as a flowmeter, after equilibration of arterial and venous flows. Venous blood is then forced by gravity through the artificially respirated lung, pressures of 40 to 60 cm of water (blood) being more than adequate for flows needed in these experiments. Oxygenated blood then flows out of the pulmonary veins, is collected in an inverted bottle, and pumped back to the dog with a Sigmamotor pump of variable speed. With the circulatory bypass system in operation as described, 12 experimental ventriculotomies have been done, under clean but not sterile conditions, during a period of 30 minutes of complete bypass of the heart and lungs, this period being preceded and followed by 2- to 3-minute periods of partial bypass. During the ventriculotomies the right ventricle was kept open for a period of 5 minutes. Continuous recordings were made of the arterial and venous pressures and usually 2 measurements were made of the blood flow. Low blood flows were used according to the azygos flow principles determined by others(3). After the experimental animal was disconnected from the circulatory bypass system the heparin in its blood was neutralized by the addition of protamine sulfate. The amount of protamine to be given was determined by dividing the total amount of heparin used by 2 and giving one cc of 1% protamine sulfate for every 10 mg of heparin. This was diluted in 100 cc of 5% glucose and given by intravenous drip over a 15- to 20-minute period. After administration of the protamine, titration tests were done to determine if sufficient quantity had been given.

Results. Twelve experimental animals have been subjected to this procedure. The first 5 animals survived the experiment without sequelae. The next 5 animals all died in the immediate postoperative period, severe bleeding being the cause of death. Despite the fact that adequate protamine had been given these dogs to neutralize the heparin by *in vitro* testing, their blood did not clot *in vivo*. In attempting to discover the cause of this reduced coagulability, it was discovered that the glass and plastic equipment that was being reused in successive experiments was not being adequately cleaned. All of this equipment was then either replaced or cleaned thoroughly with acid solution. The 2 dogs that have undergone the experiment since then have sur-

TABLE I. Blood Flow during Homologous Lung Experiments in 12 Dogs.

Survival	Flow (cc/kg/min.)	Flow (cc/min.)	Remarks
+	44–58	540–720	Aborted 1st day postop.
+	52	540	
+	36–40	540–600	
+	20–25	240–300	
+	44	600	
0	39	420	
0	44	600	
0	27	480	
0	40	420	
0	25–29	240–280	
+	33	420	
+	31–35	420–480	Ventricular fibrillation

vived without sequelae and have shown no evidence of bleeding tendency.

Summary. The use of an homologous lung is at present the simplest and most efficient method of providing extracorporeal oxygenation of blood. A simplified method of using such a lung experimentally has been described. In this experimental study of 12 animals there were 7 survivors, the 5 fatalities being caused by severe bleeding resulting from contaminated equipment.

The authors wish to express their appreciation to Mr. Dusan Surman, for his technical assistance.

1. Mustard, W. T., *et al., Surgery,* 1952, v32, 303; 1954, v36, 39.

2. Campbell, G. S., *et al.,* PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 390.

3. Andreasen, A. T., and Watson, F., *Brit. J. Surg.,* 1952, v39, 548; 1953, v41, 195.

4. Warden, H. E., *et al., J. Thor. Surg.,* 1954, v28, 331.

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Relationship of Salivary Tryptophane to Lactobacillus Colony Morphology and Dental Caries.* (22084)

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In a previous investigation of the salivary microflora of dental caries-free and caries-susceptible humans, an association of smooth colony morphology of lactobacilli with saliva of caries-free subjects has been described(1). Rough colony types predominated in saliva of the caries-susceptible subjects. The observation that a tryptone medium as well as tryptophane induced rough to smooth dissociation in colony morphology of oral lactobacilli prompted the present study of this action of tryptophane on lactobacillus colony morphology, and the possible relationship of salivary tryptophane to caries resistance.

Materials and methods. The lactobacilli used in this study were of human oral origin. The cultures were classified into 3 types, smooth, intermediate and rough on the basis of colony morphology on tomato agar. The rough colony type is flat, translucent, with an indented to serrated edge, and is stable on consecutive passage on tomato agar. Tomato juice agar was prepared according to the Hadley method(2). Rogosa's Lactobacillus Selective Medium(3), used for growth and fermentation studies, was modified for general use as a liquid medium by omission of the agar, glacial acetic acid, and 5 g of sodium acetate per liter.

To test the action of tryptophane the medium was modified to contain 10 g acid hydrolyzed Casamino Acids (Difco) instead of trypticase; 0.013 g dl-tryptophane; and 15 g agar. Additional amounts of dl-tryptophane were added as required. Modifications of the Ehrlich (paradimethylaminobenzaldehyde) and Hopkins-Cole (glyoxylic acid) tests were studied for determination of tryptophane in saliva, and the latter test was selected. The procedure has been described by Block(4), with the essential modification that the concentration of H_2SO_4 was reduced to avoid undesirable reactions with other constituents of saliva.

Three ml of resting and 3.0 ml of stimulated saliva were collected from a subject and mixed. Two ml of the mixture were placed in a 10.0 ml volumetric flask and 0.5 ml of M/25 $CuSO_4$ and 0.5 ml of glyoxylic acid were added. The flask was held in an ice-and-water bath for 5 min., then 5.0 ml of 80% H_2SO_4 were slowly added. The flask was held in the cold bath for 10 min. then placed in boiling water for 5 min. After cooling 2 min. at room temperature the contents were mixed

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by rotating the flask and cooled 8 more min. The contents were diluted to 10 ml volume with 50% H_2SO_4, incubated at room temperature for 15 min., then observed in a Coleman Junior Spectrophotometer at 515 and 570 mu. The reference standard was similarly prepared except that 0.5 ml distilled water was substituted for glyoxylic acid. The characteristic absorption maxima in this test for tryptophane and indol are respectively 570 and 515 mu. The test procedure described by Block, when applied to pure solutions of tryptophane and indol, results in colored reaction mixtures that follow the Lambert-Beer Law; concentration = –K log transmittance. Substitution of 80% H_2SO_4 introduced some error. The error in transmittance at a known concentration of 0.02 mg tryptophane was about 3%, at 0.10 mg about 15%. The values for time, volume, concentration and temperature are all critical. The subjects used as sources of saliva in this study were all young adults classified as caries-free or "immune," and caries susceptible on the basis of clinical and roentgenologic examinations. The susceptibles showed past or present caries, whereas the immunes showed no evidence either past or present.

Results. The original observation was that certain rough colony strains of oral lactobacilli exhibited a previously unobserved change to smooth colony morphology when grown on tryptone glucose extract agar. This change was demonstrable regularly on this medium, and was reversible after 2 or 3 transfers on tomato agar. The addition of 0.5% tryptone to tomato agar also induced the rough to smooth colony variation. In addition, some rough colony lactobacilli, grown on tomato agar containing 0.004% dl-tryptophane, underwent rough to smooth dissociation which did not occur on this medium without added tryptophane. However, 10 rough colony lactobacillus strains cultured on modified Rogosa's lactobacillus selective medium to which 0.0013% dl tryptophane had been added, at pH 5 and 7 showed only slight changes in colony morphology as compared to the changes observed on tomato agar. The addition of 0.02% dl-tryptophane or indol to this medium caused 7 of the strains to vary to smooth morphology. Although the results were variable, colony dissociation induced by tryptophane or indol was more common at pH 5 than at pH 7. Tryptophane and indol were about equally effective and strains of lactobacilli reacted to either, both or neither.

The rough to smooth variation was seen in all colonies which developed on media with added tryptophane, and all colonies which were picked from such media were capable of reversion. Aliquots of broth cultures developed approximately equal numbers of colonies on media with and without added tryptophane. The variation was usually reversed on first passage without added tryptophane, although some strains required 3 to 4 transfers before all smooth characteristics were lost. Successive passages on tryptophane-containing media did not render the smooth variants more resistant to reversal when removed from the influence of tryptophane. Thus the effect of tryptophane was transitory, perhaps suggestive of adaptive enzyme formation. The fermentative capacity of the smooth variants for a number of carbohydrates did not differ from the parent cultures, the original acidogenic and aciduric properties being maintained. Attempts to differentiate smooth variants from rough parents by several common capsule stains, especially by use of Alcian Blue, a carbohydrate specific stain described by McKinney(5), were not successful.

Turner and Crowell(6) have reported an association of tryptophane or tryptophane-like compounds with saliva of caries-free humans. More recently, however, Stack(7) has asserted the independence of salivary tryptophane and caries experience. In view of the above findings chemical analyses of the tryptophane and indol content of caries-immune and susceptible individuals were performed.

Descriptions of the subjects whose salivas were analyzed and results of the analyses are given in Table I. The statistical analyses of the data show a significantly greater amount of both tryptophane and indol in immune salivas. The Hopkins-Cole test is not specific

TABLE I. Analyses of Salivas for Tryptophane and Indol in 31 Subjects.

Caries immune			Caries susceptible		
Solution color	% transmission at: 570 mu	515 mu	Solution color	% transmission at: 570 mu	515 mu
Yellow	100.0	100.0	Yellow	95.0	92.2
Purple	81.3	81.3	"	99.2	97.0
"	40.0	40.0	Pink-gray	71.2	81.0
"	44.0	64.3	Yellow	94.3	95.2
"	25.3	46.0	Pink	52.1	71.2
"	28.2	41.2	Pink-brown	62.0	63.1
"	38.0	41.2	"	66.3	66.2
"	50.3	63.3	Yellow	82.1	77.1
"	36.0	58.1	Purple	53.1	88.3
"	13.1	22.0	"	56.2	106.0
"	45.0	56.1	Pink	29.3	42.2
"	34.0	61.1	"	61.1	66.3
"	51.2	57.1	"	28.0	39.2
"	28.2	50.1	Yellow	29.1	40.0
"	28.3	53.2	"	66.2	52.1
			Pink	40.0	57.2

"t" Tests

Tryptophane, 570 mu: t = 2.33 Probability = 0.038
Indol, 515 mu: t = 2.087 Probability = 0.05

Chi-Square Test for Tryptophane Color

	Purple	Non-purple	Totals
Immunes	a 14	b 1	15
Susceptibles	c 2	d 14	16
	16	15	

Chi-square = 20.26; Probability = less than 0.001

for free tryptophane alone, as combined tryptophane or compounds having similar configurations may give a color reaction. However, the deep purple color seen with most immune salivas is the same as that given by pure solutions of tryptophane.

The association of smooth colony salivary lactobacilli and high salivary tryptophane in caries immunes prompted an additional study. Several dental students having a mixed flora of salivary lactobacilli were given paraffin containing dl-tryptophane to chew intensively during 6 hours per day for one week. This procedure increased salivary tryptophane content but had no effect on the numbers of smooth and rough colony type lactobacilli isolated from their salivas.

Discussion. Dissociation of colony morphology of a culture of microorganisms due to addition of a single compound is not novel. Rogosa and Mitchell(8) have reported similar results on adding sorbitan monooleate (Tween 80) to a medium. They attributed the change in morphology to alteration of the nutritional environment. Thus, alteration of colony morphology of an entire population was not the result of genetic changes, but rather a difference in phenotypic expression of genetically similar populations in two different environments. It is rather likely that such is the case in tryptophane-induced dissociation.

The finding of significantly greater amounts of tryptophane or tryptophane-like compounds in caries-free salivas compared to caries-susceptible has confirmed the work of Turner and Crowell(6), and seems to strengthen their hypothesis that salivary tryptophane levels are related to the presence or absence of caries. It is not, however, necessarily at variance with the results of Stack (7), since a high salivary tryptophane content was found in one individual (age 14) with clinically rampant caries. In addition, Stack's conclusions might be confirmed by examining selected items of data from Table I, since his study apparently included only 3 caries-free individuals. Comparison of the results with the 2 clinically different populations in the present work, and in that of

Turner and Crowell(6), leads to other conclusions. It is also well to bear in mind that factors which may be correlated with caries immunity may be unrelated to susceptibility.

Obviously, the relationship of high salivary tryptophane or indol and the caries-immune state is not one of cause and effect. Furthermore, the rough to smooth colony dissociation of lactobacilli produced by tryptophane or indol *in vitro* is different both in extent and kind from the similar action of immune saliva(1). The latter is effective on most cultures of lactobacilli and is definitely affected by pH. It appears to be a selection process associated with inhibition of growth and results in organisms with modified physiological activities. As noted above, none of these apply to the tryptophane-indol induced variants. Likewise the low count of predominantly smooth colony lactobacilli in caries-immunes suggests that the above action of immune saliva occurs in the oral cavity, while the present findings indicate that high salivary tryptophane is not present in all immunes and does not affect the number of rough colony lactobacilli in susceptible mouths even when the salivary concentration of tryptophane is increased.

It is equally clear, however, that caries immunity and high salivary tryptophane are probably related in some way when both are present. In such cases, even the rough to smooth dissociation in lactobacillus colonies may be related to tryptophane present in immune saliva. In cases of low salivary tryptophane in immunes, this type of resistance to caries may not be involved.

Summary. 1. Addition of dl-tryptophane or indol to media induces a rough to smooth dissociation in colony morphology of some strains of oral lactobacilli. 2. This dissociation is readily reversible, is apparently not a selection, and is unaccompanied by gross changes in physiology. 3. Chemical analysis of salivas for tryptophane and indol indicates the association of greater amounts of such compounds with the caries-immune group. 4. Tryptophane-induced rough to smooth variation in lactobacilli is different from the similar dissociation which occurs in saliva of caries-immune individuals.

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1. Green, G. E., and Dodd, M. C. Accepted for publication by *J. D. Res.*

2. Hadley, F. P., and Bunting, R. W., *J. D. Res.*, 1933, v13, 198.

3. Rogosa, M., Mitchell, J. A., and Wiseman, R. F., *ibid.*, 1951, v30, 682.

4. Block, R. J., and Bolling, D., *The Determination of the Amino Acids*. Burgess Pub. Co., 1940.

5. McKinney, R. E., *J. Bact.*, 1953, v66, 453.

6. Turner, N. C., and Crowell, G. E., *J. D. Res.*, 1947, v26, 99.

7. Stack, M. V., *ibid.*, 1954, v33, 316.

8. Rogosa, M., and Mitchell, J. A., *J. Bact.*, 1950, v59, 303.

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Zone Electrophoresis of Intestinal Alkaline Phosphatase.* (22085)

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It has been shown(1) that soluble phosphatase prepared by treating the microsomal fraction of bovine intestinal mucosa with butanol traveled at a discrete peak during zone electrophoresis on filter paper, and that its solubility behavior makes it a promising starting material for further purification. Recently, Roche and Bouchilloux(2) reported purification of dog intestinal phosphatase by electrophoresis on filter paper. Morton(3) reported in detail upon application of acetone fractionation to material obtained by butanol treatment of microsomes from calf intestine. which yielded a highly purified preparation. The present paper deals with the use of electrophoresis in the starch block for purification of bovine intestinal alkaline phosphatase, and with changes in electrophoretic mobility during the purification process. Purity as great as achieved by Morton does not appear to have been reached in our study, but additional information regarding change in behavior of phosphatase during purification has been obtained.

Materials and methods. Crude intestinal phosphatase. Even with a homogenizer designed for treatment of relatively large volumes of material(4), isolation of the microsomal fraction from quantities of starting material required, was cumbersome. Consequently, butanol treatment was applied directly to the intestinal tissue. Thirty-five pounds of calf duodenum, obtained from 40 to 60 animals, were rinsed with tap water, slit, and placed in washing machine with aluminum agitator and porcelain-lined tank. Other exposed metal parts had been replaced with stainless steel and rubber with polyethylene. Five gallons distilled water and 3 gallons n-butanol were added and agitator started. After extracting 30 minutes at approximately 25°C, the intestinal tissue was removed and butanol-water emulsion transferred to glass carboys for storage at –5°C. After 12 to 18 hours, the emulsion had separated into an upper butanol layer, a middle gel layer containing much denatured protein, and aqueous layer containing the enzyme. The aqueous layer was siphoned, and pH adjusted to 4.9-5.0 with acetic acid. Precipitation and equilibration took place for 24 hours at 0°C. The supernatant solution was then siphoned and clarified by passage through a Sharples Supercentrifuge. The inactive precipitate was discarded, and an equal volume of acetone, pre-cooled to –5°C, was slowly added, with stirring, to the clarified solution. The enzyme-containing precipitate, formed upon addition of acetone, was equilibrated at –5°C for 24 hours and then removed by decantation and centrifugation at –5°C. The precipitate of crude enzyme was washed in the cold, twice with 50% acetone and twice with 100% acetone. It was then dried in a vacuum desiccator over sulfuric acid. Average yield of crude, dry enzyme was 23 g. *Starch block electrophoresis.* 1.5 g of crude enzyme were suspended in 7 ml of veronal buffer at pH of 8.6, ionic strength 0.02, and extracted with stirring for 90 minutes at 5°C. The suspension was then centrifuged in Spinco Model L preparative ultracentrifuge 45 minutes at 140000 x g, and supernatant solution recovered. This solution was subjected to electrophoresis in starch according to the method of Kunkel and Slater(5). The buffer for electrophoresis was the same as used in extraction. The starch block was 30 cm long, 5 cm wide, and one cm deep. The voltage gradient was 6.7 v/cm, and the current 7-9 ma. Migration occurred for 12 hours at 0°C. At termination of migration, the starch block was cut into 1 cm segments, each eluted by

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suspending it in 5 ml of pH 8.6 veronal buffer. Supernatant solutions obtained after centrifuging starch suspensions were used for enzyme and protein analyses. *Determination of phosphatase.* The substrate was disodium p-nitrophenyl phosphate, made as a 1% solution in 0.05 M bicarbonate buffer containing 5 x 10^{-4} M magnesium acetate, pH 10.1. Enzymatic activity was measured by incubating 2 ml of substrate solution with 0.05 ml of enzyme solution for 20 minutes at 37°C. The reaction was stopped by addition of 10 ml of 0.002 N NaOH to the reaction mixture. Absorbance of the solution was then measured at 420 mμ, setting absorbance to zero with appropriate reagent blank. Concentration of enzyme was adjusted to produce an increase in absorbance of 0.1 to 0.3. The disodium p-nitrophenyl phosphate was prepared by the method of Bessey and Love(6), and recrystallized by the method of Aschaffenburg(7). A unit of phosphatase activity is that amount of enzyme which will release one millimole of p-nitrophenol in one hour, the specific activity as the number of units per mg nitrogen. Nitrogen was determined by either micro-Kjeldahl digestion and titration, or by the ultra-micro method of Tompkins and Kirk (8). Protein in the eluates from starch blocks was measured by the method of Lowry *et al.* (9). Since color development was carried out directly on eluates, without precipitating the protein, other chromogenic material may have influenced the apparent protein distributions.

Results. The crude material used had a specific activity of 1.5, approximately 30 times greater than the average mucosal homogenate. The dried, crude material was extracted with pH 8.6 buffer, and the extract subjected to electrophoresis in starch with results shown in Fig. 1A. Positions of the peaks are not corrected for endosmotic flow. However, position of the major enzyme peak is essentially that of an immobile component. The minor peak accounts for approximately 10% of the entire enzyme found on the block. After removal from the block, the major enzyme component had a specific activity of 1.9, or 35 times that of the mucosal homogenate, and a 30-minute pre-incubation at 37°C with 0.01 M Mg^{++} and 0.01 M alanine resulted in 1.4

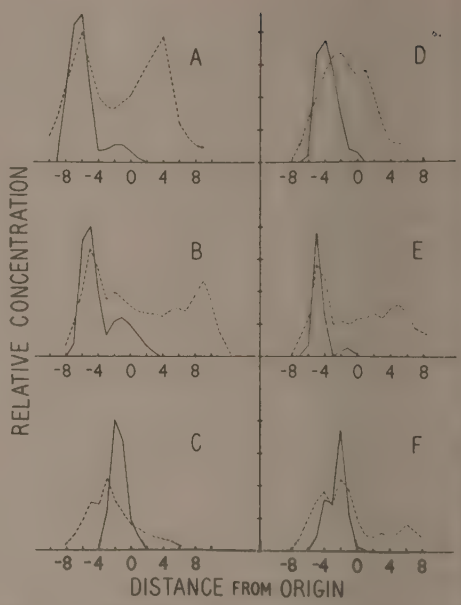

FIG. 1. Distribution of enzyme (solid line) and protein (dotted line) from starch blocks after electrophoresis for 12 hr at a voltage gradient of 6.7 v/cm and 0°C. Veronal buffer, pH 8.6, $\Gamma/2 = 0.02$. Distances from the origin (0) are indicated in cm toward the anode or −cm toward the cathode. The concentration scales are arbitrary, and not comparable for the various blocks. See text for details.

fold increase in activity over that found without pre-incubation. All specific activities are obtained after pre-incubation with Mg^{++} and alanine. The solution obtained from mid-portion of the major enzyme peak, which contained two-thirds of the total enzyme of that peak, was subjected to a second starch block electrophoresis under conditions identical with those for the first block. These results are presented in Fig. 1B. Although only the middle of the major peak found on Block 1 was used, 2 enzyme peaks were again obtained and the minor enzyme peak of Block 2 represented approximately 25% of the total. Specific activity of the major enzyme component was 4.4, indicating an 83-fold purification when compared with average mucosal homogenate. At this stage, pre-incubation with magnesium and alanine produced a 1.65-fold increase in activity. Distribution of protein on Block 2 was also unexpected, since material was found which migrated more rapidly toward the

anode than did the material on Block 1 from which it was taken. The eluate from the section of Block 2, containing the middle portion of the larger enzyme peak, was subjected to electrophoresis a third time, again using same conditions as in the first 2 blocks. These results are shown in Fig. 1C. A single enzyme peak was now found, having the mobility of what had previously been the minor component. The specific activity was 13.7, representing a 260-fold purification. Pre-incubation with magnesium and alanine produced a 6.4-fold increased activity. Protein distribution of the third starch block also differed from that observed on the 2 previous blocks. Although 2 components are discernible, the enzymatic activity does not seem to be definitely associated with either of them. It appears that enzymatically active protein on this block could not have represented more than 30% of the total. During each period of electrophoresis, 40 to 50% of the enzyme placed on the block was lost, as judged by summing enzymatic activities found in individual segments. This was a considerably greater loss than that observed in recovering enzyme added to starch. Combination of eluates from individual sections, in an attempt to reconstitute the original enzyme solution, did not produce any increase in activity over that calculated from the sum of activities in individual eluates. Since storage in solution at 0°C for the same time as required for electrophoresis produced a much smaller loss in activity, it did not appear that losses on storage and in recovery from starch, could amount to more than 15 or 20% of enzymatic activity. The larger loss during electrophoresis remains unexplained. Since the shift in mobility, accompanied by increased response to magnesium activation, suggested that tightly bound magnesium might be lost and contribute to the change in mobility, a second series of starch blocks was studied with 0.001 M magnesium acetate in the buffer. This was again 0.02 M veronal buffer at pH 8.6. The results are shown in Fig. 1D, 1E, and 1F. As before, Fig. 1D is the crude enzyme, now subjected to electrophoresis in the presence of Mg^{++}. The distribution shown in Fig. 1E is that obtained on taking the middle portion of the major enzyme component from the first block and subjecting it to electrophoresis in the presence of Mg^{++}, and the process repeated for Fig. 1F. Although presence of Mg^{++} appears to have suppressed appearance of the more mobile enzyme peak, the same general trend of results is observed as was found for electrophoresis in the absence of Mg^{++}.

Discussion. The results obtained confirm the previous conclusion 1) that alkaline phosphatase in the material obtained from n-butanol treatment of microsomes represents only a small part of the total protein. A similar conclusion can, of course, be drawn from the work of Morton(3) and the many papers of Roche and coworkers. It also serves to emphasize the problems associated with losses in activity during any purification procedures which have been investigated. Alteration in mobility during the series of electrophoretic separations indicates that purification procedures may not simply remove inactive protein, but may result in changes in the enzyme. We do not know whether a similar change would be observed during repeated fractionation with acetone. There seem to be two possible explanations for change in mobility of the enzyme. One possibility would be that the enzyme tends to form complexes with other proteins or ions which are sufficiently stable to yield discrete zones in starch block electrophoresis, but which may become less stable, as components of the original mixture are removed in electrophoretic separation. Magnesium alone is not responsible for this behavior, but magnesium does appear to have some influence on the process as judged by reduced tendency of enzyme to shift into the more mobile component in the presence of magnesium. A serious objection to this is found, however, in the behavior of the non-enzymatic protein. In general, if a complex were being disrupted and giving rise to a more highly negatively charged fraction, a somewhat comparable amount of a more highly positively charged material should be formed. This does not appear to be the case, unless such material has migrated off the block. A second possibility would be that the enzyme is altered by a process of pro-

teolysis, and that some such process is in general responsible for the fact that a portion of one block behaves so differently from what would be predicted when subjected to electrophoresis a second time. Changes in the electrophoretic behavior of enzyme preparations on standing and the degree of reproducibility in the same and different preparations do not, however, support this explanation. The problem requires further study, but we are inclined more to the view that some explanation involving dissociation of a complex provides the answer.

Summary. A crude preparation of alkaline phosphatase from bovine intestinal mucosa, obtained by treatment with butanol to release enzyme from the microsomes, was subjected to sequential electrophoresis on starch blocks. The results indicate that the phosphatase may have been present initially in a complexed form and that the complex was disrupted during repeated electrophoretic separation. Such behavior, together with the rather small amounts of enzyme protein which appear to be present in the starting material, may account for some of the difficulties encountered in attempts to purify intestinal alkaline phosphatase. An approximately 260-fold purification was achieved, but the amounts of final product obtained were too small for further study.

1. Harris, E. S., Bergren, W. R., Bavetta, L. A., and Mehl, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 593.

2. Roche, J. and Bouchilloux, S., *Bull. soc. chim. biol.*, 1953, v35, 567.

3. Morton, R. K., *Biochem. J.*, 1954, v57, 595.

4. Harris, E. S., and Mehl, J. W., *Science*, 1954, v12, 663.

5. Kunkel, H. G., and Slater, R. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 42.

6. Bessey, O. A., and Love, R. H., *J. Biol. Chem.*, 1952, v196, 175.

7. Aschaffenburg, R., *Science*, 1953, v117, 611.

8. Tompkins, E. R., and Kirk, P. L., *J. Biol. Chem.*, 1942, v142, 477.

9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *ibid.*, 1951, v193, 265.

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Effect of Season on Appetite and Food Consumption of the Lizard, *Anolis carolinensis.* (22086)

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The problem of the regulation of food intake by vertebrate animals has received much attention in recent years(1). Among the factors which seem to be implicated in appetite control are the fat stores of the animal and differences between arterial and venous blood glucose levels. Since the lizard, *Anolis carolinensis,* undergoes marked seasonal changes in total body fat as well as changes in both the concentration and distribution of other metabolites(2,3), a study of the food consumption of lizards at different times of the year was undertaken to determine if such changes in composition are correlated with variations in appetite.

Methods. Adult male specimens of *Anolis carolinensis* which had reached the plateau stage of growth were used. Colonies of 8 to 15 specimens, used to determine seasonal changes in food consumption, were formed in different months of the year from lizards recently captured in the New Orleans area. Colonies were kept for duration of each experiment (1.5 to 2.5 months) in small cages in a constant temperature (28° ± 2°C), exposed to natural hours of light (30° latitude) through a north window. An excess of meal worms, the larval form of the insect, *Tenebrio mollitor,* was kept in cages except during the 4-day fasting period preceding body weight measurements. Food intake was determined from the weight of meal worms eaten. Fortunately, large specimens of meal worms are relatively constant in gross composition if

they are well nourished and hydrated(4). Samples of meal worms were analyzed periodically and found to consist of 13% ether extractable lipid, 20% protein, 2% ash and 60% water. Using these results and the value of 2% carbohydrate obtained by Mellanby (4), the caloric equivalent of the meal worm diet was approximated as 2 kcal/g of meal worms. This value, although subject to obvious limitations, was used throughout this paper to express results obtained and to equate caloric intake with standard metabolism and fat stores of the animals. To obtain the best possible comparison of appetite in different seasons, the total food consumption during the first month in captivity was averaged and caloric intake was calculated on the basis of original body weight of the animals. Lipid content of droppings was never higher than 2% of the total caloric intake of the colony. At the end of each experiment, animals were sacrificed for organ size and composition measurements. Methods of chemical analysis were the same as those described elsewhere(3).

Results. The standard metabolic requirement of *Anolis*, as calculated from O_2-uptake studies(2), averages 24 cal/g body wt/day. Seasonal variations from the mean are of the order of 10-15%. Energy consumed in excess or deficit of the average standard metabolic requirement is shown in Fig. 1a. Appetite is much greater in summer than in other seasons. For example, animals brought in from the field in June or in July are ravenous in their hunger for meal worms. One large male caught in July consumed 1.2 g of worms within an hour after being placed in the cage. To consume a comparable amount of food, an adult man would have to eat about 40000 kcal at one meal. In contrast to the intense appetite in summer, lizards captured between October and April have little appetite for meal worms. Food eaten by these animals, even at summer temperature of laboratory, is about equal to or less than is required to meet standard metabolic requirements. It is of interest that the nitrogen contained in the food consumed by those colonies of lizards having the poorest appetite was

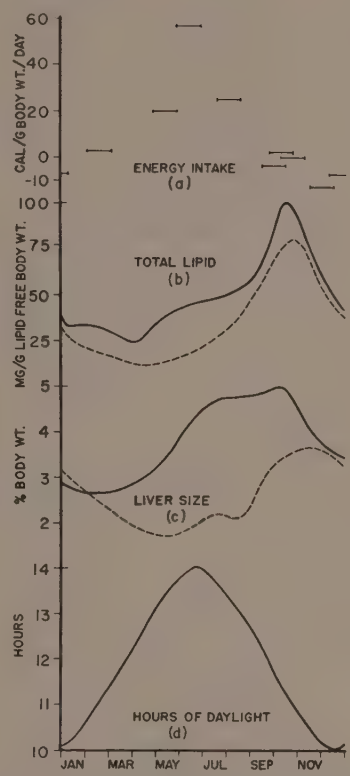

FIG. 1. (a) Seasonal fluctuations in avg energy intake of colonies of adult, male lizards kept in the laboratory at 28°C, exposed to natural hours of light. Each bar shows excess or deficit in calories eaten during the first 30 days of feeding. (b and c) compare total body lipid and liver size of animals at termination of each feeding experiment with values found for animals in the wild. Feeding ————; wild - - - - -. (d) Hours of light between sunrise and sunset at 30°N. latitude.

only slightly less than the total N losses of completely starved lizards during the same period(5). In winter lizards reach their peak food consumption 2 or 3 weeks after being brought into the laboratory, whereas animals captured during the remainder of the year exhibit their greatest appetite during the first few days of exposure to laboratory conditions.

In Fig. 1b total lipid content of captive animals is compared with that of lizards in the wild. During months of intense appetite, lizards increase in body weight and in total lipid content. The fat storage process occurs throughout the summer but appears to be

most efficient during late summer and fall. The time lag between peak appetite and maximum fat deposition may be due to differences in the activity of *Anolis* in these two seasons(6). Calories consumed in excess of standard metabolic requirements exceeded greatly the caloric equivalent of fat stored by mid-May to mid-July captive colony but were only slightly greater than the caloric equivalent of fat stored by mid-July to October colony. From the time of maximum fat storage in the fall until mid-January, appetite is minimal and animals undergo a progressive decrease in body weight and in fat content. Although fat stores have diminished considerably by late December, appetite remains poor. Apparently, in this season, food consumption by the lizard is not directly correlated with lipid stores.

In Fig. 1c liver size of captive lizards after their average 2-month feeding period is compared to liver size of lizards in the wild(2). In late spring, with the return of appetite, the liver enlarges. This enlargement is relatively rapid in animals in captivity where the food is readily obtained and temperatures are always warm. The increase in size of the organ involves a growth as well as an increase in fat. Liver nitrogens of lizards from feeding colonies sacrificed between January and April averaged 0.717 ± 0.151* mg liver N/g lipid free body wt, but by June the average was 0.976 ± 0.159* mg liver N/g lipid free body wt. Apparently, once appetite returns, one of the first important changes in gross composition of the animal is synthesis of liver protein. Once liver protein reaches this peak value it does not vary greatly until the period of hibernation in early winter. The size of livers of captive lizards throughout the year is generally greater than size of organ of animals in the field. As the maximum liver protein in captive lizards in autumn is only slightly greater than the maximum value found in wild controls, the larger liver of captive lizards in this season is due primarily to an increased fat content of the organ.

Discussion. Besides *Anolis*, the lizard, *Eumeces fasciatus*(7), and the alligator(8) are known to have a diminished appetite during the winter. Since *Anolis* does not eat well when brought from the field into a warm laboratory in winter, factors other than mere temperature differences are involved in appetite control in this animal. The possibility that daylength may be such a factor is suggested by a comparison of the chart of energy intake during the different seasons with Fig. 1d which shows the hours of light between sunrise and sunset at different times of the year(9). Preliminary measurements of food consumption of experimental groups exposed to short and long periods of light substantiate this impression(10). It is highly possible that light as well as season of year are further factors which must be considered in any appraisal of the mechanism of appetite control in certain animal forms.

Summary. Food consumption of adult male lizards (*Anolis carolinensis*) at 28°C is maximal in summer and minimal in winter. This seasonal variation in appetite appears to be correlated with hours of daylight and is responsible in part for seasonal changes in body composition of this lizard.

* Standard deviation from the mean.

1. *Ann. N. Y. Acad. Sci.*, 1955, v63, 1.

2. Dessauer, H. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 351.

3. ———, *J. Exp. Zool.*, 1955, v128, 1.

4. Mellanby, K., *Proc. Roy. Soc., B*, 1932, v111, 376.

5. Dessauer, H. C., and Farmer, C. J., unpublished observations.

6. Greenberg, B., and Noble, G. K., *Physiol. Zool.*, 1944, v17, 392.

7. Fitch, H. S., *Univ. Kansas Publ. Mus. Nat. Hist.*, 1954, v8, 126.

8. McIlhenny, E. A., *The Alligator's Life History*, Christopher, Boston, Mass., 1935.

9. Berry, F. A., Bollay, E., and Beers, N. R., *Handbook of Meteorology*, McGraw-Hill, New York, N. Y., 1945, 930.

10. Dessauer, H. C., and Fox, Wade, unpublished observations.

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Blood Serum Xanthine Oxidase of Rats Poisoned with Carbon Tetrachloride. (22087)

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In previous studies(1,2) it has been shown that carbon tetrachloride when injected subcutaneously increases the activity of rat liver xanthine oxidase. A strong activation was also observed when this drug was added *in vitro* to the liver homogenate. We were therefore interested to know if the same activation could be detected in the blood serum. Few reports have been published on presence of xanthine oxidase in blood. Older literature dealing with distribution of this enzyme in tissues, is deficient in quantitative data on blood xanthine oxidase(3,4). Blauch *et al.* studying uric acid of rat blood observed that when rat blood is incubated with xanthine an increase of uric acid is obtained due to presence of xanthine oxidase(5). Using a spectrophotometric technic Westerfeld *et al.* determined xanthine oxidase activity of rat blood serum in experimental cancer of the liver and reported values of 50-60 μg of xanthine oxidized/1 ml/hour(6).

Due to the lack of quantitative values for xanthine oxidase and xanthine dehydrogenase of blood serum measured by manometric and colorimetric methods we undertook a series of determinations in normal rats which served for comparison with the injected ones.

Methods. Three hundred male Wistar white rats weighing between 100 to 150 g and maintained on well balanced diet were used. Blood samples were withdrawn by

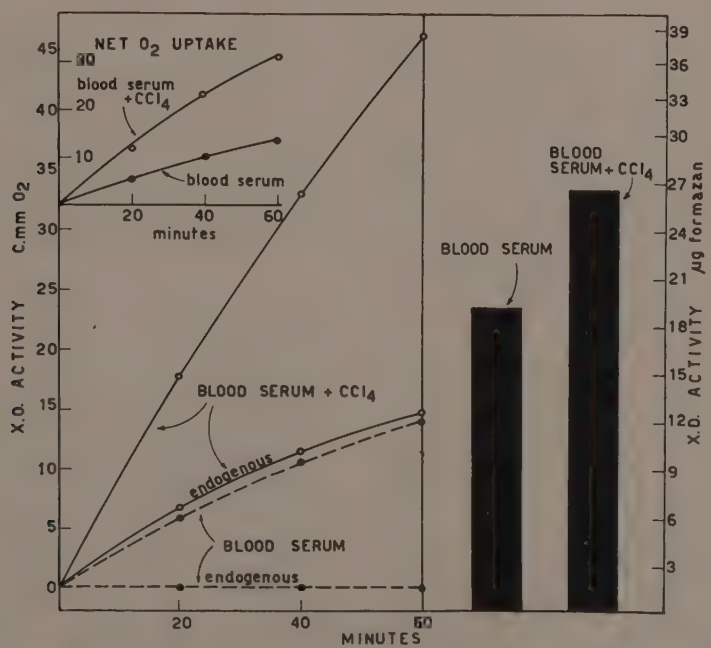

FIG. 1. *In vitro* effect of CCl_4 on xanthine oxidase and xanthine dehydrogenase activity of rat blood serum. Left: oxygen uptake of blood serum in mm^3 measured in Warburg manometers before and after addition of CCl_4. Net oxygen consumption due to xanthine is shown in upper left-hand corner. Right: xanthine dehydrogenase expressed in μg formazan produced/1 ml serum/30 min. before and after activation by CCl_4.

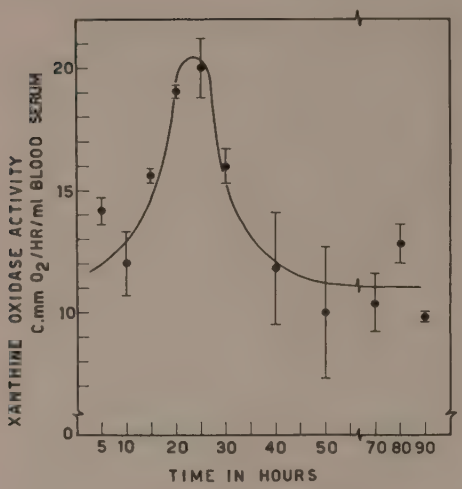

FIG. 2. Blood serum xanthine oxidase activity of rats poisoned with CCl_4. Each point in the graph represents determinations on pooled nonhemolyzed serum from 15 rats (3 groups of 5 rats). Vertical bars indicate avg values ± stand. dev.

heart puncture and each determination was carried out on pooled nonhemolyzed serum from 5 rats. The animals were injected subcutaneously with 0.1 ml CCl_4/100 g body weight and the blood taken at different intervals (5, 10, 15, 20, 25, 30, 40, 50, 70, 80 and 90 hours) after injection. The coagulated blood was centrifuged, the serum separated and used immediately for analysis. Hemolyzed samples were discarded since it was observed that hemolysis interferes with reduction of the triphenyltetrazolium dye. Xanthine oxidase activity was measured manometrically as previously described(1) using 1 ml of blood serum, 0.2 ml of 0.05 M xanthine solution in 0.05 M NaOH and enough pyrophosphate buffer pH 8.6 to give a final volume of 2.0 ml. Xanthine dehydrogenase activity was measured colorimetrically by the method of Villela(7) using 1 ml of blood serum, 0.1 ml of 0.05 M xanthine solution, 0.3 ml of 0.1% triphenyltetrazolium chloride and 2.0 ml of pyrophosphate buffer pH 8.6. *In vitro* experiments were carried out by adding 0.1 ml CCl_4 to 10 ml blood serum, mixing well for 10 minutes and then centrifuging. Determinations were made in the CCl_4 free supernatant layer.

Results. Normal values were obtained from the blood serum of 55 rats. The average values together with the range plus or minus the standard deviation showed values of 12.9 ± 0.86 cmm O_2/ml serum/60 minutes for xanthine oxidase activity and 17.81 ± 3.00 μg formazan/ml serum/30 minutes at 37°C for xanthine dehydrogenase activity. Each determination was carried out on pooled serum from 5 rats.

In vitro activation of rat blood serum xanthine oxidase and xanthine dehydrogenase by CCl_4 is shown in Fig. 1. Values of 120% activation for xanthine oxidase and 38.5% for xanthine dehydrogenase activity were observed.

The enzyme activities increased in animals injected with CCl_4, the highest values were found 20-25 hours after injection of the drug (Fig. 2). Thereafter, the enzyme activity for xanthine oxidase and xanthine dehydrogenase returned to the normal level.

Discussion. Bruns and Neuhans showed that aldolase and phosphohexose isomerase increased in serum of mice poisoned by intraperitoneal administration of carbon tetrachloride(8). The highest activity was reached 18 to 20 hours after injection of the drug. However, the same enzymes decreased in liver, reaching lowest values 50 hours after injection. These authors suggested that a loss of these enzymes from liver tissues could be the cause of the sharp increase presented in the serum.

It was demonstrated that CCl_4 activates strongly the xanthine oxidase of the rat liver a few hours after subcutaneous injection of the drug(1). Xanthine oxidase was also activated in the blood serum 20 hours after the single subcutaneous injection of carbon tetrachloride as shown in Fig. 2. The simultaneous increased activity of this enzyme in the liver and blood serum indicate that a different mechanism than that proposed for aldolase and phosphohexose isomerase must be responsible.

The distribution of xanthine dehydrogenase was recently studied in the serum of normal rats. It was found that enzyme activity is confined to the lipoprotein fractions(9), sug-

gesting that the activation by CCl_4 could be attributed to the split of the linkages between the enzyme and the lipids as observed when milk(10) and liver xanthine oxidase(2) are treated by other organic solvents.

Summary. Quantitative determinations of xanthine oxidase and dehydrogenase activity in blood serum of normal rats were made. It was shown that a single injection of 0.1 ml CCl_4/100 g body weight is able to activate the blood xanthine oxidase and dehydrogenase, reaching the highest values 20-25 hours after injection. However, after 35 hours the values returned to normal. Activation of the enzyme was also obtained *in vitro* by adding CCl_4 to a sample of serum of known enzyme content.

1. Villela, G. G., and Mitidieri, E., *Nature,* 1955, v175, 208.
2. ———, *Rev. Bras. Biol.,* 1955, v15, 285.
3. Morgan, E. J., *Biochem. J.,* 1926, v20, 1282.
4. Dixon, M., *Enzymologia,* 1938, v5, 198.
5. Blauch, M. B., and Koch, F. C., *J. Biol. Chem.,* 1939, v130, 455.
6. Westerfeld, W. W., Richert, D. A., and Hilfinger, M. F., *Cancer Res.,* 1950, v10, 486.
7. Villela, G. G., *Rev. Bras. Biol.,* 1954, v14, 455.
8. Bruns, F., and Neuhaus, J., *Arch. Biochem. Biophys.,* 1955, v55, 588.
9. Mitidieri, E., Ribeiro, L. P., Affonso, O. R., and Villela, G. G., *Biochim. et Biophys. Acta,* 1955, v17, 587.
10. Polonovsky, M., Baudu, L., and Neuzil, E., *Bull. Soc. Chim. Biol.,* 1947, v29, 958.

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Recently Classified Types of Coxsackie Virus, Group A. Behavior in Tissue Culture. (22088)

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In addition to the 10 previously described types of Coxsackie virus, Group A(1,2), 9 new types have now been assigned numbers. The representative strain of Type 11 is the Belgium 1 strain of Godenne and Curnen (3), that of Type 12, the Texas 12 of Contreras, Barnett, and Melnick(4). Type 13 was isolated in this laboratory from fecal specimens from Mexico. Type 19 was received from Dr. R. J. Heubner, and the remainder from Dr. J. H. S. Gear of the South African Institute for Medical Research(5). All are viruses which induce flaccid paralysis in suckling mice but no apparent disease in older mice and are characterized by the production of marked muscle degeneration but no significant lesions in the central nervous system in the experimental animal. With the exception of Types 12 and 14 all resemble Types 1, 7, and 9 in low infectivity of brain suspensions for suckling mice. Passage and testing with muscle suspension are necessary. In neutralization tests in suckling mice there is definite, although moderate, reciprocal cross reactivity of A-11 with A-15 and of A-13 with A-18. As noted by Contreras(4), A-12 cross reacts with A-5. Regular cross reactions may also be found with A-8 and A-12, more observable when brain tissue is used in neutralization tests. Slight cross reactivity of A-14 in A-7 serum, A-15 in A-17 serum, and A-17 in A-11 serum has been seen.

Tissue culture. Methods. Human uterine tissue and trypsinized monkey kidney tissue cultures were prepared as previously described (6). Trypsinized monkey testicular cultures were made similarly to the kidney cells. HeLa cell cultures were supplied by the Laboratories for Virology under the direction of Dr. Irving Gordon. On receipt, the nutrient fluid containing human serum was removed and replaced by 1.5 ml of the maintenance fluid of Scherer, Syverton, and Gey(7) without added serum. The tubes were rotated so that the entire inside surface was washed and were then allowed to stand upright for a minimum of one-half hour. The washings were removed and replaced by 1.0 ml of mainten-

TABLE I. Cytopathogenicity of Coxsackie Virus.

Coxsackie virus		Monkey cells trypsinized		Human cells	
Group	Type	Kidney	Testes	Uterine plasma clot	HeLa cells
A	9	+	+	+	—
	11	—	—	+	+
	13	—	—	+	+
	15	—	—	+	+
	18	—	—	+	+
B	1	+	+	—	+
	2	+	+	—	*
	3	+	+	+	+
	4	+	+	—	(+)
	5	+	+	+	+

* Only one strain regularly cytopathogenic under the given conditions.
(+) More erratic than B-1, 3, and 5.

ance fluid. All types not cytopathogenic for these cells were retested on HeLa cells that had been given two further washings of 1.0 ml each. No additional cytopathogenic strains were encountered. In early tests 10% chicken serum was used in the maintenance fluid for the test and later, 2% calf serum. For many of the results recorded no serum was added. Three tubes each were inoculated with 0.1 ml of the test material. For the cultures grown on glass the inoculum was added to the tube after the maintenance fluid; for plasma clot cultures the tissue was inoculated and let stand at room temperature for one-half hour before the addition of the maintenance fluid. Neutralization tests were made, in general, as previously described(6).

Results. Trypsinized monkey kidney cell cultures. All types of Group B were cytopathogenic for monkey kidney cells, but only Type 9 of Group A(8,9). None of the newer Group-A types induced evident cell damage (Table I).

Trypsinized monkey testicular tissue cultures. In previous tests on stationary plasma clot cultures of monkey testicular tissue with 3 strains of A-7, 2 of A-9, one each of B-1, B-4, and B-5, and 6 of B-2, cytopathogenic activity was obtained with the A-9 strains only. Steigman obtained cell damage with 2 strains of B-2 on roller tubes of plasma clot cultures with fluid 199 as maintenance medium(10). Using the trypsinized cells, we have found that the same types cytopathogenic for monkey kidney cells induce cell damage in the testicular tissue grown on glass. With the Group-B types the effect was more prompt and more marked. The newer Group-A types were not cytopathogenic.

Human uterine tissue (plasma clot). Among the previously numbered types Group A, Type 9, some strains of Group B, Type 3, and Type 5(6) were found to be cytopathogenic for human uterine tissue. Four of the new types in Group A, Types 11, 13, 15, and 18, are also cytopathogenic. Cell damage may appear in from 3 to 7 days but is usually definite at 5 days. All are neutralized by their specific immune sera. Two of 4 strains of A-14 caused minor cell damage in early passages on this tissue and also on plasma clot cultures of monkey testicular tissue. This, however, could not be established on passage.

HeLa cells. Our experience with the previously numbered types has been, in general, similar to that of Crowell and Syverton(11). Group A, Types 1-10, were not cytopathogenic. Strains of Group B, Types 1 and 3, are cytopathogenic, as is Type 5. More difficulty was experienced with B-4. In earlier trials reactivity was erratic but more recently we have been able to make type determinations of a number of strains on HeLa cells, although

TABLE II. Neutralization Test in HeLa Cell Tissue Culture Coxsackie Virus, Group A, Type 15.

Test dose			A-15 No. 52108	
Virus	Serum Kind	Serum Dilution	5 days	6 days
10^{-2}	Maintenance medium		2+ 2+ 2+	4+ 4+ 4+
10^{-3}			2+ 2+ 2+	4+ 4+ 4+
10^{-4}			— ? —	2+ 4+ 2+
10^{-5}			— — —	— — —
10^{-1}	Normal hamster	5	2+ 2+ 2+	3+ 4+ 4+
	A-11	5	— ? —	1+ 2+ —
		10	— — 1+	2+ 2+ 3+
		20	2+ 2+ 2+	4+ 4+ 4+
	A-15	10	— — —	— — —
		50	— — —	— — —
		160	— — —	— — —
		640	2+ 1+ ?	4+ 3+ 3+
Uninoculated controls			— — —	— — —

same as control uninoculated tissue; ? slight damage, specificity doubtful; 1+ = small but definite area of damage; 2+ = large areas of definite damage but with large remaining areas of normal cells; 3+ = extensive destruction; 4+ = complete destruction.

endpoint titers are not high. With B-2 only one strain of 10 tested was consistently cytopathogenic on HeLa cells. Two other strains continue to give erratic results on repeated transfer. Among the new Group-A types the 4 which are cytopathogenic for human uterine cells, Types 11, 13, 15, and 18, are also cytopathogenic for HeLa cells. Beginning cell damage may be seen in from 2 to 6 days. Satisfactory neutralization tests may be made with these strains. The cross reactivity noted in the mouse neutralization tests is also evident in tissue culture (Table II).

Summary. Nine additional types of Coxsackie virus, Group A, have been assigned numbers, Types 11-19. Strains of 4 of these types, Types 11, 13, 15, and 18 are cytopathogenic for human uterine tissue in plasma clot culture and for HeLa cells, but not for trypsinized monkey kidney or testicular cells.

1. Dalldorf, G., *Ann. N. Y. Acad. Sci.*, 1953, v56, 583.

2. Melnick, J. L., *ibid.*, 1953, v56, 587.

3. Godenne, M. O., and Curnen, E. C., *Acta Paediat., Belg.*, 1954, v8, 29.

4. Contreras, G., Barnett, V. H., and Melnick, J. L., *J. Immunol.*, 1952, v69, 395.

5. Gear, J. H. S., and Measroch, V. In: *Poliomyelitis*, Papers and discussions presented at 2nd International Poliomyelitis Conference, p437. (J. B. Lippincott Co., Philadelphia, Pa., 1952)

6. Sickles, G. M., Feorino, P., and Plager, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 22.

7. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

8. Robbins, F. C., Enders, J. F., Weller, T. H., and Florentino, G. L., *Am. J. Hyg.*, 1951, v54, 286.

9. Riordan, J. T., Ledinko, N., and Melnick, J. L., *ibid.*, 1952, v55, 339.

10. Steigman, A. J., School of Medicine, University of Louisville, Ky., personal communication, Dec. 15, 1954.

11. Crowell, R. L., and Syverton, J. T., *J. Immunol.*, 1955, v74, 169.

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Association of I^{131}-Labeled Lactogenic Hormone with Cytoplasmic Nucleoprotein of Mammary Gland Cell.* (22089)

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It has been reported from this laboratory (1) that lactogenic hormone labeled with I^{131} becomes associated predominately with the cellular particulates following intraductal injection into the rabbit mammary gland. It was suggested that this study indicates the importance of the mitochondria and microsomes in the synthetic activity associated with milk secretion and that the site of action of the lactogenic hormone at the cellular level is in association with the mitochondria and microsomes of the secretory cells. Studies of the nature of this association of the lactogenic hormone with the mitochondria and microsomes would be expected to yield information basic to an understanding of the mode of action of the lactogenic hormone at the cellular level. To this end, studies of the chemical nature of this association have been carried out.

Due to the nucleoprotein and lipoprotein nature of the cellular particulates this investigation has been limited to study of the involvement of two chemical moieties, the pentosenucleic acid and the lipid, in the association of the lactogenic hormone with the mitochondria and microsomes. Demonstration of the involvement of particulate lipid or nucleic acid in the lactogenic hormone-particulate association would at least indicate something of the nature of this association and to that

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extent add to the understanding of the basic biochemical mechanisms involved.

Methods and materials. The method which was used for the labeling of the lactogenic hormone with radioactive iodine has been described(1). Experimental treatment of the animals and preparation of the samples for counting of sample radioactivity were also described(1). For all studies reported here an isotonic sucrose homogenate of the mammary gland from pseudopregnant rabbits, which had been injected intraductally with radioactive iodine labeled lactogenic hormone was centrifuged at 1000 g for 10 minutes to remove nuclei and the supernatant from this centrifugation was used for fractionation. Grinnan and Mosher(2) used 4 M guanidine hydrochloride for the production of a highly polymerized and apparently undenatured RNA from liver. This same procedure has been used in this study for the precipitation of nucleoprotein from the mammary homogenate. A second procedure for precipitation of nucleic acids and nucleoproteins has been studied by Berkman, Housewright and Henry (3) and Euler and Heller(4). These studies indicated that streptomycin was effective in precipitation of cytoplasmic nucleoproteins. In the present work the homogenate, as prepared above, was brought to pH 7.0-7.4 and streptomycin added to a final concentration of M/200 to M/400, held at room temperature for one hour with occasional shaking, chilled to 0°C for one hour and the nucleoprotein precipitate removed. Two methods which have been used for the solubilization of particulate proteins were studied for their action on the lactogenic hormone-particulate association. Wainio *et al.*(5) studied the solubilizing effect of two to four percent desoxycholate on the cytochrome oxidase associated with the insoluble particulates of heart muscle preparation. Morton(6) studied the effect of excess n-butyl alcohol on the solubilization of enzymes associated with insoluble cytoplasmic particulates. The isotonic sucrose homogenate with the nuclei removed was treated for three hours at room temperature with these reagents, centrifuged at 25000 g for 90 minutes, dialyzed against distilled water and the streptomycin precipitable material counted as being the material taken into solution by these procedures. The results obtained with these methods are of interest due to the apparent dependence of these methods on the removal of lipids and the rupture of lipoprotein bonds.

TABLE I. Precipitation of Activity from Unfractionated Particulate Preparations.

4M guanidine HCl precipitation			Streptomycin precipitation		
Ppt., cpm	S'nat., cpm	% ppt.	Ppt., cpm	S'nat., cpm	% ppt.
243867	120070	67.0	476015	113406	80.7
71531	53388	57.2	206666	35995	85.1
76482	29284	72.3	111549	14886	88.2
206240	36873	84.8	82860	10702	88.5
601242	115590	83.8			
375056	137538	73.1			
1850	1556	54.3			
27806	4988	84.7			
152759	11921	92.7			
Avg % ppt.	74.4 ± 14.2			85.6 ± 10.3	

Results. The studies reported here for the distribution of radioactivity following precipitation of the nucleoprotein with guanidine hydrochloride or streptomycin indicate that the cytological localization of the lactogenic hormone noted previously is, in fact, an association of the lactogenic hormone with the nucleoprotein of the cytoplasm.

These data (Table I) indicate that precipitation with 4M guanidine hydrochloride precipitates approximately 75% of the lactogenic I^{131} hormone as nucleoprotein. Approximately 85% of the lactogenic I^{131} is precipitated with streptomycin. It is seen that the agreement between the two methods is rather good, with at least 75% of the radioiodine activity labeling the lactogenic hormone being precipitated as the nucleoprotein by these methods.

The experiments using desoxycholate or n-butyl alcohol in attempts to solubilize the labeled lactogenic hormone-particulate association have given essentially negative results (Table II). These data, while not extensive, indicate by their negative nature that the particulate lipids are probably not involved in the hormone-particulate association.

Discussion. Basic to an understanding of

TABLE II. Solubilization of Particulates as Measured by I^{131} Activity of Non-Dialyzable, Streptomycin Precipitable Material.

	4% desoxycholate	Excess n-butyl alcohol	
	No. 1	No. 1	No. 2
Cpm soluble	17760	9450	7953
Cpm unfractionated particulate preparation	423620	423620	292035
% soluble	4.2	2.2	2.7
% insoluble or dialyzed	95.8	97.8	97.3

the mode of action of a particular hormone will be information which indicates where and how the metabolism of the target cell is controlled by the hormone. These studies appear to implicate the pentosenucleoprotein of the cytoplasmic particulates as being important in the action of the lactogenic hormone on the secretory cells of the mammary gland. Little is known of the function of the nucleic acid and nucleoprotein of the cytoplasm but it has appeared that they are involved in the synthetic processes of the cell, particularly those in which the cytoplasmic particulates are involved. It may be postulated that the association of the lactogenic hormone with the cytoplasmic particulate nucleoprotein results in changing the conditions of enzyme attachment to the particulate nucleoprotein or in changing the activity of the enzymes which are already in association with the nucleoprotein. Such changed conditions of enzyme attachment, affecting the enzymatic composition or activity of the cytoplasmic particulates, could result in changing the direction of the synthetic activity of the cell as occurs with the initiation of lactation.

Summary. Studies have been carried out with lactogenic hormone labeled with radioactive iodine in pseudo-pregnant rabbit mammary gland which indicate that the labeled hormone becomes associated with the cytoplasmic particulate nucleoprotein. Other data suggests that the particulate lipids are probably not involved in the lactogenic hormone-particulate association. These findings have been discussed in terms of their relation to the shift in synthesis which occurs in the mammary gland with the initiation of lactation.

1. Williams, W. F., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 524.
2. Grinnan, E. L., and Mosher, W. A., *J. Biol. Chem.*, 1951, v191, 719.
3. Berkman, S., Housewright, R. D., and Henry, R. J., *J. Immunol.*, 1949, v61, 349.
4. Euler, H., and Heller, L., *Arkiv. Kemi, Mineral., Geol.*, 1948, v26a, 14.
5. Waino, W. W., Cooperstein, S. J., Kollen, S., and Eichel, B., *J. Biol. Chem.*, 1948, v173, 145.
6. Morton, R. K., *Nature*, 1950, v166, 1092.

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Carbon and Water Contents of Mouse and Rat Tissues. (22090)

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In reporting tissue analyses for radiocarbon, tracer chemists have employed a wide variety of units (*e.g.* per cent of injected dose, specific activity of tissue carbon, total activity per organ, activity per unit weight of fresh or dried tissue, etc.). Sometimes the choice is clearly dictated by the immediate context of the measurement; at other times the units are those most conveniently derived from the chosen method of radioassay (*e.g.* specific activity when $BaC^{14}O_3$ is assayed at "infinite thickness"). In analyzing and comparing data from several published sources, the reader must frequently translate from one set of units to another. Adequate information, however, is seldom presented for even an approximate conversion.

Table I records soft-tissue concentrations

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of water and of organic carbon in mice and rats from this laboratory. The data were obtained in the course of a study on the metabolism of C^{14}-labelled atropine(1). All values were presumably influenced by the genetic and nutritional histories of the test animals. Insofar as these mice and rats are representative of those in general laboratory use, the information in Table I makes it possible to transform, among the following reference systems, any C^{14} concentrations which are known in any one of these systems: activity per mol of tissue C, per unit weight of wet (fresh) tissue, or per unit weight of dried tissue. Unlike the water data, the tissue carbon values are thought to be unique in terms of their accuracy and precision (*cf.* 2), illustrating that the carbon method described by one of us(3) is particularly useful for tissue samples.

Materials and methods. The data were derived from 12 male white mice of mixed breed, ranging in body weight from 26.8 to 38.7 g (mean 33.3 g), and from 12 male albino rats with body weights between 106 and 153 g (mean 128 g). The rats were obtained from a colony bred in these laboratories(4) from an original Wistar strain. After weaning, both mice and rats were maintained on a Purina Rat Chow plus cooked fresh meat and mixed grain (corn and wheat, with oats as a binder) 3 times weekly. They were deprived of food for 24 to 48 hours before sacrifice, but water remained available *ad libitum*. Some of the animals received atropine one-half hour to 24 hours before sacrifice, but these injections had no apparent effect on the results reported here. Animals were killed by chloroform inhalation, and selected tissues were removed promptly for weighing and desiccation. As soon as each organ was dissected free, a weight was quickly obtained on a torsion or knife-edge microbalance. Because evaporation was not controlled, weights of the smaller organs (*e.g.* adrenals, gall bladder) were unreliable, and consequently no values of moisture content are presented for these organs. All tissue samples were dried *in vacuo* (pump operating continuously) over Drierite for a period of 24 hours at room temperature.

TABLE I.* Carbon and Water Contents of Mouse and Rat Tissues.

	Water (% wet wt)		Carbon (% dry wt)	
	Mouse	Rat	Mouse	Rat
Adrenals	—	—	66.2	58.4
Gall bladder & bile	—	—	56.9	—
Pancreas	—	71.6	55.0	53.7
Skin (hair sheared)	—	65.5	54.6	53.7
Spinal cord†	70.7	71.6	59.3	56.9
Medulla & brain stem†	73.3	77.1	55.5	57.1
Cerebellum†	76.8	78.4	55.6	54.1
Cerebrum†	77.2	78.8	54.2	54.5
Liver	70.5	75.6	53.5	52.2
Blood (heart)	—	—	52.6	51.1
Kidneys	74.7	75.7	52.1	51.0
Thymus	76.1	—	53.6	—
Genital apparatus‡	—	75.4	52.2	51.0
Submaxillary gland	—	76.9	52.8	48.6
Parotid gland	—	78.2	—	48.9
Urinary bladder	70.8	74.0	54.2	52.9
Diaphragm	76.4	76.8	52.3	50.4
Heart	75.5	78.2	51.8	49.6
Hamstring muscles	77.0	77.6	51.2	48.4
Stomach§	78.4	78.9	51.8	49.9
Colon & caecum§	79.5	79.4	51.3	49.7
Duodenum & jejunum‖	77.4	77.5	49.5	48.9
Ileum‖	78.2	76.4	48.5	48.5
Lungs	78.3	79.7	51.6	49.4
Spleen	75.8	77.7	49.6	48.6
Testes	82.0	86.2	50.0	48.1
Colonic contents (feces)	—	72.5	37.9	30.5

* Conventional methods (5) were used to calculate stand. deviations for determinations of carbon and water in mice and rats. These 4 stand. deviations were found unexpectedly to be almost equal, namely about 2% units. Because almost every value in the table is based on 12 animals, the estimated stand. error for each of the means is 0.6. The stand. error of the difference between two means in the same column is approximately 0.8.

† The rat data (only) represent here 5 analyses each (stand. error of the difference between means about 1.3).

‡ Genital apparatus included epididymi, vasa deferentia, seminal vesicles, and part of the prostate.

§ Stomach, colon, and caecum were emptied of contents and mucosal surface was gently rinsed with isotonic saline.

‖ Analyses of duodenum, jejunum, and ileum included entrapped secretions and small amounts of chyme.

Only negligible losses of water could be detected during additional periods of desiccation. After reweighing, the specimens were ground to fine powders; aliquots weighing 2 to 25 mg were subjected to high-temperature combustion and analysis by the method of Gabourel, Baker, and Koch(3). Standards (*e.g.* CP sucrose) were burned daily to check the carbon analyses.

Results. Tissue analyses are summarized by the means in Table I. Almost every mouse tissue had a slightly lower water content and higher carbon content than the corresponding rat organ. Within each species, tissues rich in carbon (*e.g.* near the top of Table I) were generally poor in water; the converse was equally true.† From this inverse correlation and from the observation that tissues of grossly fat mice were invariably richer in carbon than those of lean mice, we infer that variations in tissue carbon reflect chiefly differences in the tissue lipid content, relative to the stores of protein and carbohydrate. This impression is supported by the invariably high carbon level in brain (lipid-rich) and in bile and adrenal gland (sterol-rich). Of course any material with a large inorganic ash is correspondingly deficient in carbon (*e.g.* feces). Analyses on gross subdivisions of the central nervous system suggest that concentrations of carbon and water vary progressively and reciprocally along the cerebrospinal axis. Probably these variations among portions of the brain and cord parallel the ratio between carbon-rich white matter and water-rich grey matter.

† Because carbon is expressed only in terms of the dry-tissue weight, this inverse correlation is not fatuous.

Summary. Soft-tissue concentrations of organic carbon and of water were measured in male rats and mice. Both the method and results (Table I) are thought to be useful to the radiocarbon biochemist.

Authors are grateful to Dr. Lee S. Crump of Department of Radiation Biology at University of Rochester for statistical analysis of the data.

1. Gosselin, R. E., Gabourel, J. D., Kalser, S. C., and Wills, J. H., *J. Pharmacol. Exp. Therap.*, 1955, v114, 217.

2. Skipper, H. E., Bryan, C. E., While, L., and Hutchison, O. J., *J. Biol. Chem.*, 1948, v173, 371.

3. Gabourel, J. D., Baker, M. J., and Koch, C. W., *Anal. Chem.*, 1955, v27, 795.

4. Gray, E. LeB., and Derum, J. W., *Chem. Corps Med. Lab. Research Rep.* No. 365, Army Chemical Center, Md., May, 1955.

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Analysis of Micro Quantities of Sweat for Sodium Chloride by Conductivity Method.* (22091)

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The collection and analysis of small quantities of sweat is an important tool in assessing the mineraloid activity of the adrenal cortex. However, the small amounts which are collected by the most practical technics(1) make analysis of the diluted material by flame photometry or chemical means quite difficult. Even if meticulous care is taken to thoroughly clean glassware, small amounts of sodium are extracted from the glass itself(1). To prevent this, the glassware must be silicone coated and the water freshly distilled. Special precautions must be taken in the handling of the unknowns. This imposes further limitations on the value of the sweat test for clinical and investigative use. To obviate the difficulties inherent in the chemical analysis of such dilute quantities of sodium, the use of radioactive sodium has been suggested(2). The concentration of chloride in the sweat increases with the rate of sweating and parallels the concentration of sodium. Either chloride or sodium can be used as a measure of adrenal cortical activity in the absence of an abnormal salt intake. The potassium concentration falls as the perspiration rate increases(3). But the relationship of the fall of potassium to the rise in chloride is a linear one. Because of the linear relationship between the excretion of sodium, potassium and chloride, it seemed feasible to

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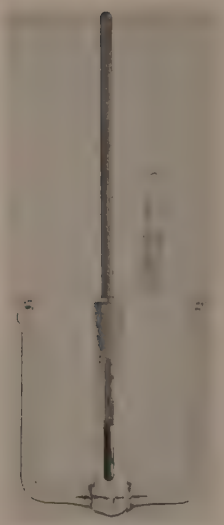

FIG. 1. Henry cell with small volume capacity. Wire leads are placed into side arms filled with mercury.

measure total electrolytes by the conductivity method and express them as NaCl since this element constitutes well over 90% of the total electrolyte concentration of rapidly secreted sweat.

Method. Conductivity was measured by a resistance measuring device which is modification of the Wheatstone Bridge.† This instrument has a sensitivity capable of determining one part of sodium chloride in two million parts of water. A special Henry type conductivity cell was constructed with a volume of only 5 cc total (Fig. 1).‡ It has a cell constant of approximately 0.1. It is cleaned with detergent and rinsed with distilled water. It is dried by washing with alcohol and ether or acetone. There is no necessity to obtain highly purified distilled water since the initial conductivity of the water is determined and is subtracted from the conductivity of the water after the sweat has been introduced. This difference is due to conductivity of the sodium chloride in the sweat itself. We have found that more stable readings can be obtained when the water in the cell initially contains a very small amount of sodium chloride. Also, the solution in the Henry cell can be used over again after making one determination since the blank reading is subtracted from the final reading. If the same solution is used over again, care must be exercised lest the concentration of electrolyte exceed the range of the cell being used. Sweat is collected in a 10 λ pipette and diluted with 5 cc of distilled water in the Henry cell. The content of the pipette is thoroughly transferred by rinsing with the distilled water in the cell. A Henry cell has the advantage over a dip type of cell in that it allows for complete transfer without spillage. The dilution technic described allows for a final accuracy within 3% if the blank value for the distilled water is subtracted.

Results. Utilizing the same dilution (1/500 as given above) a chart can be constructed which relates the observed conductivity of the diluted material to the actual concentration of sodium in the sweat. Known standards of sodium chloride were prepared and diluted 1/500. The initial conductivity of this water

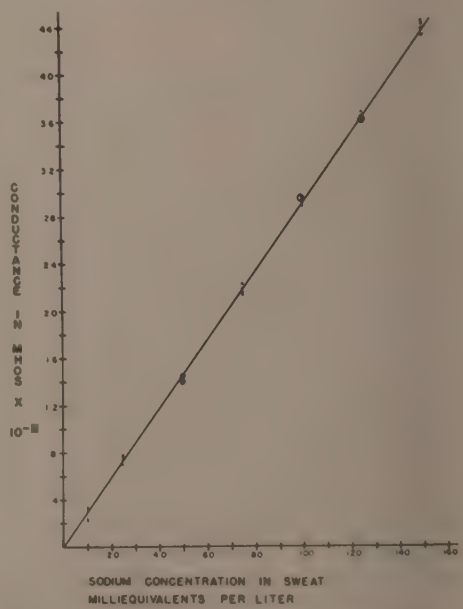

FIG. 2. Relationship between conductivity in Mhos and sodium chloride concentration of 1/500 diluted sweat. Initial (blank) conductivity of water has been subtracted from observed value.

† Leeds Northrup Model No. 4866.

‡ Made for us by Delmar Scientific Laboratories, Chicago, Ill.

was subtracted from the final reading so that this increased conductivity measured in Mhos was directly related to the sodium chloride concentration. Fig. 2 was prepared from determinations of known concentrations of sodium chloride. There is a linear relationship between conductance§ and sodium chloride concentration.

The standard error was calculated from 20 determinations on a standard solution containing 125 me/L. The dilutions were made with the micropipette in a manner identical to the handling of actual sweat. The deviations of these determinations were used to calculate the standard error of the mean. The standard error of the mean was 3.8 x 10^{-7} Mhos which corresponds to less than 4 milliequivalents. These determinations were done with a dip cell. The accuracy has been further increased by the use of the Henry cell.

§ Conductance is the reciprocal of ohms and is expressed in mhos.

Summary. The fact that the ionic content of rapidly secreted sweat consists almost entirely of sodium chloride make feasible use of conductivity measurement to determine the sodium chloride content of micro quantities of sweat. This method has a high degree of accuracy and is at the same time simple and rapid.

1. Dole, V. P., Stall, B. G., and Schwarts, I. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 412.
2. Decker, B., Genkius, G., Braunwold, E., *ibid.*, 1955, v88, 60.
3. Locke, W., Talbot, N. B., Jones, H. S., and Worcester, J., *J. Clin. Invest.*, 1951, v30, 325.

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A Method for Collection of Micro Quantities of Rapidly Secreted Local Sweat.* (22092)

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In many diseases it is recognized that disturbances in salt and water metabolism occur(1). It is not known whether the salt retention or losses which are seen as manifestations of some diseases are caused by variations in the minute to minute blood levels of mineraloid hormone activity. These gaps in our information arise from the fact that there is no simplified clinical and investigative tool for testing the overall activity of the salt hormones of the adrenal cortex and other organs. Urinary hormone assays of aldosterone can at best measure only gross changes which occur over a 24-hour period. It is a painstaking laboratory examination which can be done only in a few specially equipped research laboratories. The demonstration by Conn(2) that the concentration of sodium or chloride in perspiration mirrors the mineraloid activity of the adenal cortex provided the basis for the achievement of such a practical test. Most investigators have used hot rooms and other forms of thermal stimuli to produce diaphoresis, and have collected the sweat from an extremity in a water tight envelope made of plastic or thin rubber. The difficulties inherent in this method of sweat collection are obvious. Aside from the difficulties of collection, the electrolyte concentration of sweat has been shown to be dependent upon the rate of sweating. For this reason, it is best to measure the electrolyte concentration at the maximal rate of sweat production(3). It is also essential to be able to take frequent collections of small samples which are unmixed with a large dead space volume of a different concentration. Dole *et al.*(4) have devised a small chamber which contained an absorbent filter paper. Sweat was induced in the skin under the chamber by the injection of a parasympatheticomimetic drug in this area. While this has greatly simplified the collection of

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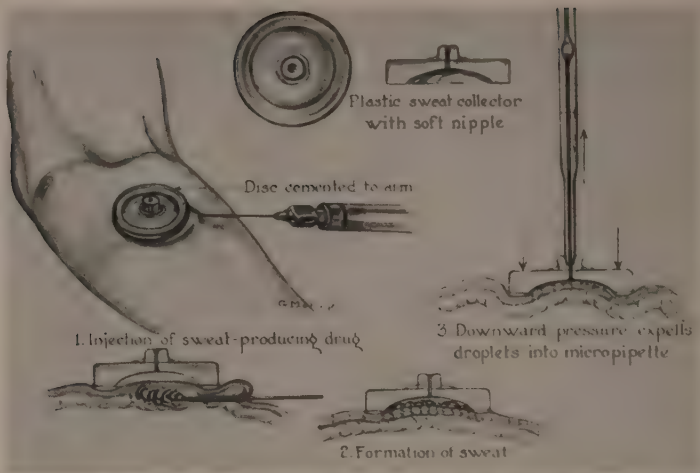

FIG. 1. Demonstration of manner of induction and collection of small quantities of sweat. The design is somewhat different than illustrated in the top center cross-section above. The walls of the cavity are convex rather than concave.

small amounts of sweat, only 10 mg of sweat are collected in about 20 minutes. Even with the best precautions in handling, and with precautions in the prevention of evaporation, it is difficult to achieve a high degree of accuracy when handling such minute quantities. The filter paper is then pulped to extract the sodium from it and hence a high degree of dilution of the collected sweat is required.

Materials and methods. Sweat is induced by local injection of cholinergic drugs. Although we originally used only acetyl beta methyl choline the solution suggested by Dole is excellent. Its formula has been slightly modified by the use of saline rather than distilled water in a more convenient amount: Acetyl beta methyl choline (Mecholyl), 25 mg; procaine, 20 mg; sterile isotonic salt solution, 10 ml. Sweat is collected in a small round plastic disc with a central cavity. A capillary hole communicates with the center of the concavity. The diameter of the concavity is one inch and its depth ⅛ inch. This covers a collection area of 5 cm^2 of skin when the collection chamber is cemented onto the skin. The sweat which collects in the concavity can be seen through the transparent plastic, but is protected from evaporation until collection is desired. On the outer flat surface a small circular piece of rubbery vinyl chloride plastic (Tygon or Koroseal) is cemented so that it coincides with the capillary hole in the lens. The completed assembly is shown in Fig. 1. The design now used is slightly different than illustrated. With concave dome-like collection chamber as illustrated, the sweat and bubbles were sometimes trapped at the periphery of the chamber by occlusion of the capillary hole by the skin. If the sweat collecting chamber has converging (convex) side walls, this does not occur. The vinyl chloride plastic teat is made to accommodate the tip of a λ pipette (λ = .001 ml). The type of pipette best suited for this purpose is one which is calibrated to contain rather than to deliver the required amount. The pipette is then washed out with the diluting fluid to make certain the transfer is complete. For routine work 10 λ pipettes are used although they may be purchased in any size up to 500 λ. Graduated pipettes are ideal for determining secretory rates. Although not recommended for this use, ordinary hemoglobin pipettes are 20 λ (20 cmm or .02 ml) and some have graduations at 10 λ and 20 λ.

Sweat is collected from a smooth and flat flexor surface of an extremity or more usually the back. If there is abundant hair, the site should be first shaved. The skin is then cleansed with a 1/1000 solution of aqueous

zepharin and thoroughly rinsed with distilled water. The skin is then dried by blotting and rubbed off with acetone. This process degreases the skin and allows for better adhesion of the plastic disc. The chamber is cemented onto the skin by moistening the outer edge of the disc with "Duco Cement." When the rim is tacky (not fluid), it is pressed against the prepared skin site and held for 3 to 5 minutes.

After fixation of the disc, the skin outside the disc punctured with a fine gauge 1½-inch needle, the point of which is directed toward the center of the disc 0.5 ml of the diaphoric substance is injected subcutaneously (Fig. 1, 1,) with a tuberculin syringe. Local diaphoresis is rapid and copious. In 5 minutes an excess of 20 λ has usually been secreted.

When it is desired to collect the sweat, the arm is placed so the air bubble is near the center of the disc. The tip λ pipette is engaged with the vinyl plastic bottom and gentle downward pressure exerted on the disc. This causes the skin to bulge into the sweat collecting chamber and expresses the sweat into the pipette (Fig. 1, 3). It is best to slightly over fill the pipette and to remove the excess fluid from the tip by capillary action. If this is done, the tip should be wiped with a silk rag rather than filter paper or cotton which may be too highly absorbent and remove the excess too rapidly. Automatically self-filling pipettes are also available from most laboratory supply houses. The sweat is then transferred to the dilution fluid and prepared for flame photometry, or other chemical analysis. We have used the conductivity method to determine sodium. It is described in the preceding paper.

Summary and conclusion. A simplified method of collecting and measuring small quantities of rapidly secreted sweat is described. This method employs local chemical induction of sweating and the collection of this sweat in a hollowed out plastic chamber. The sweat can be transferred to a λ pipette by expressing the sweat through a capillary orifice in the chamber.

1. Overman, R. R., *Physiol. Rev.*, 1951, v31, 285.
2. Conn, J. W., *Arch. Int. Med.*, 1949, v83, 416.
3. Locke, W., Talbot, N. B., Jones, H. S., and Worcester, J., *J. Clin. Invest.*, 1951, v30, 325.
4. Dole, V. P., Stall, B. G., and Schwartz, I. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 412.

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An Antimalarial Effect *in vitro* of Parapyruvic Acid. (22093)

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When preparations of the bird malaria parasite *Plasmodium lophurae* were removed from their host duck erythrocytes and maintained extracellularly *in vitro,* their survival and development were favored by the addition to the culture medium of both adenosinetriphosphate (ATP) and sodium pyruvate (1,2). It was noted that a commercial preparation of sodium pyruvate did not have the favorable effect obtained with sodium pyruvate prepared in the laboratory(3). This fact, combined with the recent isolation by Montgomery and Webb(4) of an impurity present in some samples of sodium pyruvate, which inhibited the tricarboxylic acid cycle in isolated mitochondria, suggested an investigation of the possible antimalarial effects of this impurity. Two samples of this material, identified as a lactone of parapyruvic acid (the dimer of pyruvic acid), have been obtained through the kindness of Dr. Carmel Montgomery. They have been tested against *P. lophurae* maintained *in vitro* both extracellularly and intracellularly, as well as against the infection *in vivo.*

Materials and methods. The lactone of parapyruvic acid was dissolved in water (redistilled in a Pyrex glass still), and the solu-

tion was brought to pH 6.8 with 1 N NaOH and diluted to give a concentration of 15 mg/ml. The solution was sterilized by filtration through an ultrafine glass filter, tubed in small amounts, frozen and stored at –20°C. Freshly thawed material was appropriately diluted and used for the experiments. Since it was added to cultures having a pH of 6.8 which were subsequently held at least 1 day at 40°C, it is probable that the lactone was soon converted to parapyruvate, the form found by Montgomery and Webb to be effective in inhibiting mitochondrial respiration. For experiments with *extracellular* parasites, blood from a duckling heavily infected with *P. lophurae* was treated with guinea pig complement and an anti-duck erythrocyte serum prepared in rabbits(2). This resulted in a mixture containing free parasites, free red cell nuclei and clumps of agglutinated, mostly hemolyzed erythrocytes, both with and without parasites. A purified suspension containing only free parasites and free red cell nuclei was obtained by removing the agglutinated cells by means of a 40-second low speed centrifugation (to a maximum speed of 400 rpm). The parasite suspension was added, in amounts of 0.6 ml for purified suspensions and 0.3 ml for unfractionated suspensions, to 50 ml flasks lined with a thin plasma clot(3) and containing 3.3 ml of culture medium. The medium was a concentrated extract of duck erythrocytes prepared in the manner previously described(1) and containing all of the supplements of known chemical nature which have been found(5) to favor the extracellular survival of these intracellular parasites (yeast adenylic acid, coenzyme I, ATP, coenzyme A, malate and pyruvate). Appropriate small volumes of suitable dilutions of the stock solution of parapyruvate were added to some of the cultures. The flasks were incubated on a rocker at 40°C, and a current of air with 5% CO_2 was passed through them.

An initial Giemsa-stained film was prepared from the parasite suspension used as inoculum. After one day of incubation samples were taken from each flask and used for the preparation of Giemsa-stained films and wet mounts. The latter were examined with a phase contrast microscope(1). The stained films served for counts of the proportions of degenerate(1) parasites. For experiments with parasites developing *intracellularly* in suspensions of intact duck erythrocytes the medium and methods were those previously found to support multiplication of *P. lophurae*(6,7). Each culture flask held 6 ml of a suspension containing about 1,000,000 red cells per cu mm, with about 50 parasites per 1000 red cells. The parapyruvate was again added as small volumes of appropriate dilutions of the stock solution. The flasks were incubated on a rocker at 40°C, and a current of air with 5% CO_2 was passed through them. Giemsa-stained films were prepared from each flask initially (Day 0) and after 1 and 2 days of incubation. These were used to count the parasites per 1000 red blood cells and the proportions of parasites in different stages of development.

TABLE I. Effect of Parapyruvate on Extracellular Survival *in vitro* of *Plasmodium lophurae*. Culture medium was a duck erythrocyte extract prepared as previously described, except in Exp. A where the extract was diluted with isotonic diluent to one-third its usual strength. A purified suspension of extracellular parasites served as inoculum in Exp. A-D. In Exp. E an unpurified mixture of hemolyzed, agglutinated erythrocytes and free parasites was used.

Exp.	Flasks	Parapyruvate added, mg/ml	% degenerate parasites in stained films made after 20 hr at 40°C
A	1, 2	0	3, 2
	3, 4	.85	32, 25
B	1, 2	0	3, 2
	3, 4	.40	14, 15
C	1, 2	0	10, 9
	3, 4	.40	20, 20
	5, 6	.04	20, 23
	7, 8	.008	12, 19
D	1	0	1
	2	.4	13
	3	.04	6
	4	.008	1
E	1, 2	0	1, 1
	3, 4	.08	4, 5
	5, 6	.025	7, 4
	7, 8	.008	2, 3

Results. The deleterious effect of the parapyruvate preparation on the extracellular survival of *P. lophurae* is clearly shown by the

TABLE II. Effect of Parapyruvate on Development of *P. lophurae* in Suspensions of Duck Erythrocytes Maintained *in vitro*, in 8 Flasks.

Parapyruvate added, mg/ml	% young parasites (uninucleate, not pigmented) on day: 0	1	2	Total parasites/1000 red blood cells on day: 0	1	2
0	48	0	68	50	52	59
0	74	0	68	52	51	51
.075	52	0	24	52	50	18
.075	64	0	36	48	33	29
.025	48	2	60	48	42	20
.025	56	0	64	41	31	21
.008	72	0	68	50	43	26
.008	80	0	74	68	82	20

experiments summarized in Table I. Concentrations as low as 40 and 25 μg per ml were definitely harmful to the parasite, whereas a concentration of 8 μg per ml appeared to be at the border line of activity. The relative degrees of degeneration seen in the stained films were likewise seen in the fresh preparations examined by phase contrast.

The parapyruvate similarly had a deleterious effect on the development of the parasites within erythrocytes, as illustrated by the experiment shown in Table II. It will be seen that all 3 concentrations tested reduced the number of parasites present after 2 days' incubation to about half that of the control flasks. Some reduction in parasite numbers was already evident at the two higher concentrations after one day's incubation. It is worth noting that in the flasks with the highest concentration (75 μg/ml) the proportion of young parasites which reinvaded erythrocytes on day 2 was much less than in the control flasks or in those with lower concentrations.

Discussion. The inhibition of respiration of isolated mitochondria by parapyruvate appears to depend on competition between this compound and alpha-ketoglutarate for alpha-ketoglutaric acid oxidase(4). Since there is good evidence for the participation of the tricarboxylic acid cycle in the oxidative metabolism of malaria parasites(8), it may well be that the deleterious effect of parapyruvate on the parasites is likewise a result of interference by this compound with the utilization of alpha-ketoglutarate. In the experiments with extracellular parasites this effect occurred at concentrations down to 1.5 x 10^{-4}M in the presence of pyruvate at a concentration of 5 x 10^{-3}M. In the experiments with parasites developing intracellularly the effect was marked even at the lowest concentration tested of 5 x 10^{-5}M. Perhaps the absence of added pyruvate and the longer period of incubation in the latter experiments both contributed to this greater effect.

The ability of the parapyruvate to penetrate infected duck erythrocytes and reduce the numbers of *P. lophurae* developing intracellularly suggested that the compound might have some antimalarial action *in vivo*. This did not prove to be the case. Ducklings infected with *P. lophurae* were treated once on the 3rd day and twice on each of the 4th and 5th days of their infection with doses of 2.5 to 10 mg parapyruvate per 100 g body weight, administered in solution intravenously. No effect on either the course of the infection or the ducklings was observed. Presumably the parapyruvate was altered before it could exert its action on the malaria parasites.

Summary. When the erythrocytic forms of *Plasmodium lophurae* were maintained extracellularly *in vitro* the addition of parapyruvate at concentrations down to 25 μg per ml increased the proportion of degenerate parasites present after one day's incubation. In cultures with intracellular parasites in suspensions of intact duck erythrocytes, the addition of the parapyruvate at concentrations down to 8 μg per ml decreased sharply the numbers of parasites present after two days' incubation. Somewhat higher concentrations also interfered with the reinvasion of erythrocytes by merozoites formed in such cultures. No antimalarial effect was observed when the compound was administered intravenously to ducklings infected with *P. lophurae*.

1. Trager, W., *J. Exp. Med.*, 1950, v92, 349.
2. ———, *Some Physiological Aspects and Consequences of Parasitism*, (W. H. Cole, Ed.), Rutgers University Press, 1955, 3.
3. ———, *Ann. N. Y. Acad. Sci.*, 1953, v56, 1074.
4. Montgomery, C. M., and Webb, J. L., *Science*,

1954, v120, 843.
5. Trager, W., *J. Exp. Med.*, 1952, v96, 465.
6. ———, *J. Parasitol.*, 1947, v33, 345.
7. McGhee, R. B., and Trager, W., *ibid.*, 1950, v36, 123.
8. Speck, J. F., Moulder, J. W., and Evans, E. A., Jr., *J. Biol. Chem.*, 1946, v164, 119.

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Paradoxical Retention of Uric Acid by Uricosuric Drugs in Low Dosage.* (22094)

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It has long been known that the renal excretion of uric acid in man is increased by large doses of salicylate and suppressed by small doses(1,2). Recent, more adequately documented studies(3) showed that acetylsalicylic acid in 5.2 g daily oral dosage caused a mean increase of 37.2% in the 24-hour urinary uric acid output of 15 subjects, with a concomitant mean fall of 25.5% in plasma uric acid, whereas 2.0 g daily dosage resulted in a mean decline of 14.4% in the urinary excretion and 7.5% rise in the plasma concentration of uric acid. Renal clearance studies indicate that this reversible effect is related to the concentration of salicylate in blood plasma(4) or, more precisely, in the urine(3); high salicylate concentrations cause an increase in the renal clearance ratio, $C_{uric\ acid}/C_{inulin}$, low salicylate levels depress this ratio.

Our data illustrate these relationships in respect to salicylate and indicate further that the reversibility of effect on uric acid clearance can be demonstrated, by appropriately designed experiments, to occur with all other uricosuric drugs we have examined, namely, phenylbutazone, phenylbutazone analogs, and probenecid. It would appear that the capacity to cause uric acid retention when given in low dosage is, to greater or lesser degree, a property common to uricosuric agents. The observation may have implications of interest in relation to the still obscure nature of renal tubular transport systems for uric acid.

Methods. The experiments were performed in gouty subjects in the intercritical phase of the disorder. Standard renal clearance technics(5) were employed throughout, with minor modifications described elsewhere(6). The general experimental design was to obtain 3 or 4 pre-medication urine collection periods for estimation of the inulin and PAH clearances, then to infuse the drug to be studied at a slow rate yielding low initial plasma drug concentrations, and gradually to increase the rate of drug infusion to attain uricosuric plasma drug levels. The 6-10 urine collection (catheterization) periods obtained during infusion of the drug in each experiment were each of 10-30 minutes duration; urine samples (spontaneous voidings) collected in some instances after termination of the drug infusion represented 2-hour intervals during the day and 4-hour intervals at night. Blood samples were procured at the midpoint of each urine collection period. In the salicylate infusion experiments, 4 g sodium salicylate dissolved in 500 ml isotonic saline solution was injected at a rate of 12-15 mg salicylate/min. for one or more periods, with an increment of 5-10 mg/min. in each urine collection period thereafter. In 2 phenylbutazone experiments, the infusing solution contained 2 g phenylbutazone/250 ml isotonic saline solution, was delivered at a rate of 12 mg/min. for 2 clearance periods and at double the rate thereafter. In the 3 remaining phenylbutazone, the 2 G-25671 and 6 of the 8 phenylbut-

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TABLE I. Representative Experiments Showing Effect of Slow Infusion of Sodium Salicylate, Phenylbutazone and Probenecid on Uric Acid Clearance.

Case	Period (min.)	Rate of drug infusion (mg/min.)	Plasma drug conc. (mg %)	Plasma uric acid (mg %)	$C_{uric\ acid}$ (ml/min.)	$\frac{C_{uric\ acid}}{C_{inulin}}$ (%)
J. S.	Control	0	0	11.5	13.9	10.7
	"	0	0	11.5	14.9	11.7
	"	0	0	11.5	14.4	11.2
		Sodium salicylate infusion (4 g/500 ml saline sol.)				
	15	12	Trace	11.4	13.9	10.7
	13	12	1.5	11.3	7.95	6.36
	11	17	3.1	10.9	7.08	5.66
	11	20	5.8	10.8	9.58	7.92
	12	25	9.0	10.9	13.4	11.4
	12	29	12.0	10.9	18.0	14.4
	12	34	15.0	10.8	21.6	18.6
	10	40	17.1	10.6	27.2	23.6
	11	45	21.0	10.7	31.5	26.5
L. R.	Control	0	0	9.1	8.05	8.47
	"	0	0	9.1	8.09	8.00
	"	0	0	9.2	8.02	8.41
		Phenylbutazone infusion (2 g/250 ml saline sol.)				
	15	12	2.3	9.5	6.15	6.73
	16	12	3.0	9.4	6.04	6.65
	15	24	10.0	9.5	10.4	10.6
	15	24	12.0	9.5	12.7	14.3
	15	24	15.5	9.3	13.9	18.0
	10	24	16.7	9.0	24.4	23.4
R. G.	Control	0	0	7.3	8.73	6.35
	"	0	0	7.2	9.14	5.53
	"	0	0	7.0	8.07	6.52
		Probenecid infusion (0.1 g/500 ml 5% glucose sol.)				
	15	.2		7.4	8.07	5.08
	15	.2		7.6	5.46	4.48
	19	.2		7.2	6.15	4.70
	16	.4		7.0	7.17	5.48
	16	.4		6.9	7.63	5.26
	13	.4		7.0	8.03	5.11
	19	.6		7.1	7.42	5.30
	20	.6		7.0	8.45	6.12
	23	1.2		7.0	10.6	6.80
	17	1.2		7.0	12.0	8.17

azone metabolite 1 experiments, the initial infusion rate was 1.4 mg/min. with an increase of 1.4-2.0 mg/min. in each following collection period. In 2 of the metabolite 1 experiments cited the drug was infused rapidly in a total dosage of 8 mg/kg body weight. The details of the probenecid injections are given in the text. Uric acid was determined by a modification of the method of Buchanan *et al.* (7) incorporating the use of uricase, urea cyanide-carbonate and arseno-phosphotungstic acid, inulin by the Schreiner method(8), creatinine by the method of Bonsnes and Taussky(9), salicylate by the method of Brodie *et al.*(10). For the plasma phenylbutazone, G-25671 and metabolite 1 determinations we are indebted to Dr. J. J. Burns.

Results. Salicylate. Rapid infusion of sodium salicylate at a rate sufficient to maintain plasma total salicylate concentrations at 15-34 mg % effected an increase in $C_{uric\ acid}/C_{inulin}$ from a mean of 5.5% in the control clearance periods to a mean of 20.6% in 6 subjects so studied(3). The experiment included in Table I (Case J.S.) illustrates the effects on $C_{uric\ acid}/C_{inulin}$ of a slow, sustained infusion of sodium salicylate delivered at a rate permitting gradual increase of plasma total salicylate from trace levels to 21 mg %. In the 3 control periods, $C_{uric\ acid}/C_{inulin}$ varied from 10.7 to 11.7%, and remained within this range in the initial period of the infusion. At a

TABLE II. Summary of Slow Infusion Experiments with Uricosuric Drugs, Showing Maximal Suppression of Uric Acid Clearance at Low Plasma Drug Concentrations.

	Case	Drug infused	Plasma drug conc. (mg %)	$C_{uric\ acid}/C_{inulin}$ Control (%)	Minimum (%)	Change (%)
1.	M. L.	Salicylate	2.2	7.22 ± .29	3.38	−53.2
2.	J. S.		2.8	11.2 ± .41	5.66	−49.5
3.	S. K.		4.7	5.14 ± .14	2.80	−45.6
4.	M. M.		1.8	5.48 ± .33	3.10	−43.5
5.	M. S.		4.1	6.40 ± .72	4.11	−35.8
6.	J. B.		8.4	5.16 ± .21	4.38	−15.8
1.	L. O.	Phenylbutazone	2.1	5.73 ± .15	3.48	−39.3
2.	N. P.		4.0	1.87 ± .24	1.22	−34.8
3.	J. S.		2.6	5.24 ± .55	4.20	−19.8
4.	L. R.		3.0	8.30 ± .46	6.65	−18.9
5.	J. B.		1.3	5.15 ± .29	4.72	+ 8.4
1.	M. G.	Metabolite 1	.5	4.97 ± .31	3.34	−32.8
2.	J. Z.		3.6	4.72 ± .25	3.22	−31.8
3.	G. H.		7.0	3.79 ± .13	2.85	−24.8
4.	M. R.		5.9	6.60 ± .17	5.15	−22.0
5.	H. B.		4.3	4.92 ± .35	3.90	−20.7
6.	J. B.		1.3	8.94 ± .14	6.99	−20.7
7.	N. K.		5.0	3.72 ± .33	3.05	−18.0
8.	I. S.		.4	6.98 ± .17	7.67	+10.0

plasma total salicylate concentration of 1.5 mg % $C_{uric\ acid}/C_{inulin}$ was depressed to 6.36%, and persisted well below the premedication ratio until the plasma total salicylate reached 9 mg %, at which point $C_{uric\ acid}/C_{inulin}$ returned to the control limits. At plasma total salicylate levels of 12 mg %, $C_{uric\ acid}/C_{inulin}$ exceeded the premedication ratios and continued to rise with further augmentation of the plasma drug concentration, ultimately reaching 26.5% at a plasma total salicylate level of 21 mg %. In Table II, the pertinent results of 6 such salicylate infusion experiments are recorded. It will be noted that a (transitory) decline in $C_{uric\ acid}/C_{inulin}$ occurred in each instance, the peak decline varying from 53.2-15.8% of the mean premedication figures. In five of these subjects the decline exceeded 30%.

Fig. 1 illustrates an experiment (Case M.L.) in which the plasma total salicylate again was slowly raised by infusion of sodium salicylate, the infusion was then discontinued, and the plasma drug concentration decay curve followed. It will be noted that $C_{uric\ acid}/C_{inulin}$ was depressed at low plasma total salicylate levels in both the initial ascending and terminal descending segments of the curve representing plasma drug concentrations. Distinct uricosuria occurred in the intervening period of plasma total salicylate levels in excess of 10 mg %.

Phenylbutazone. Rapid infusion of phenylbutazone in 10 subjects consistently caused a rise in $C_{uric\ acid}/C_{inulin}$, from a mean control value of 6.0% to a mean peak of 19.5% (11). The effects of a slow, sustained infusion are illustrated in Table I (Case L.R.). $C_{uric\ acid}/C_{inulin}$ fell initially, from a mean control figure of 8.3% to 6.7% at low plasma phenylbutazone concentrations, then rose to uricosuric levels as the plasma drug concentration exceeded 10 mg %. The results of 5 such experiments are summarized in Table II; in 4 a transitory decline in $C_{uric\ acid}/C_{inulin}$ was detected, the peak decline in 2 instances exceeding 30%. Unlike the experience with salicylate, observations for 24-72 hours following termination of infusion of phenylbutazone (11) failed to reveal any post-medication suppression of $C_{uric\ acid}/C_{inulin}$. This is attributed to the slow rate of biotransformation of phenylbutazone (approximately 20%/day) (12) and the consequent persistence of high plasma and urinary drug concentrations (11).

Phenylbutazone analogs. Phenylbutazone metabolite 1 (p-hydroxyphenylbutazone) (13), which exhibits uricosuric activity approximating that of phenylbutazone in equiv-

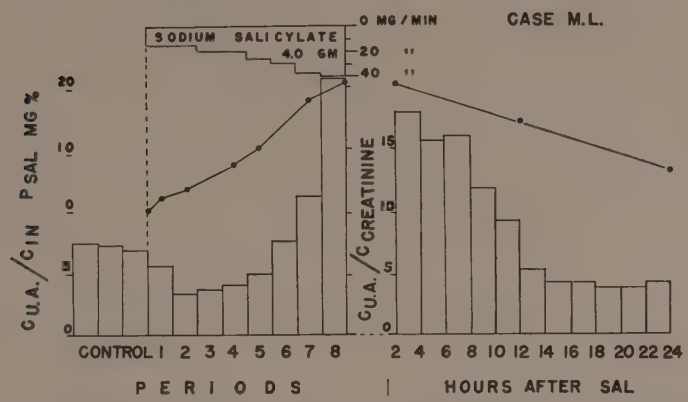

FIG. 1. After 3 control periods, sodium salicylate was infused slowly and plasma total salicylate level gradually raised (linear curve, top). Infusion was discontinued after eight 20-min. periods. Effects of rising and falling plasma drug levels on $C_{uric\ acid}/C_{inulin}$, respectively, $C_{uric\ acid}/C_{creatinine}$ are indicated by the bars (see text).

alent dosage, at low plasma drug levels caused a transitory decline in $C_{uric\ acid}/C_{inulin}$ in 7 of 8 experiments (Table II). In 2 instances the decline exceeded 30%. G-25671, a phenylbutazone derivative in which the butyl side chain is replaced by a phenylthioethyl group, is a more potent uricosuric agent(12). In 7 rapid infusion experiments, G-25671 caused a mean 5-fold increase in $C_{uric\ acid}/C_{inulin}$, from a mean pre-medication value of 6.15% to a mean peak of 30.6%(14). In 2 slow infusion experiments, a transient, moderate decline in $C_{uric\ acid}/C_{inulin}$, reaching –16.1% at its peak, was observed at a plasma G-25671 level of 0.6 mg %; at plasma drug levels of 1.0 mg % uricosuria was already in evidence.

Probenecid. Suppression of $C_{uric\ acid}/C_{inulin}$ could be demonstrated with this potent uricosuric agent(6) only by slow infusion of very small quantities of the drug. Injection at the rate of 0.2 mg probenecid/min. gave a maximum decline in $C_{uric\ acid}/C_{inulin}$ of 27% below the mean ratio of the control period (Case R.G., Table I); at 0.3 mg probenecid/min., the maximum decline was 14.6%; at 0.4 mg/min. suppression of uric acid clearance was no longer demonstrable; and at higher drug infusion rates uricosuria set in promptly.

Discussion. The observations herein recorded may explain, in part, some of the conflicting results reported in the literature, notably in respect to phenylbutazone. This drug has been found by most investigators to increase urinary excretion of uric acid in ordinary therapeutic dosages by mouth, but by others to have no effect or, indeed, to cause retention of uric acid. This confusion is compounded by the fact that phenylbutazone consistently lowers the serum uric acid level even in instances without uricosuria. This discrepancy has been interpreted to imply suppression of uric acid formation by phenylbutazone but direct measurement of the rate of uric acid biosynthesis, using N^{15}-labeled glycine in subjects receiving the drug, fails to support this view(15). The evidence now available indicates that such discrepant declines in serum uric acid are apt to be factitious, reflecting hemodilution due to renal retention of sodium and water caused by phenylbutazone(11,14). Further clarification of this relationship has been achieved by renal clearance studies, including those reported here, which reveal the sequence of renal effects at rising plasma phenylbutazone levels, corresponding to various dosages of phenylbutazone. Appreciable retention of salt and water may begin in association with plasma phenylbutazone levels as low as 1 mg % and continues at higher drug levels(11,14); suppres-

sion of uric acid clearance occurs within an intermediate range of plasma phenylbutazone concentration; and distinct uricosuria begins at plasma phenylbutazone concentrations of about 10 mg %, becoming more marked at higher drug levels(11,14,16). These observations explain how, without suppression of uric acid biosynthesis, administration of phenylbutazone may cause lowering of the serum uric acid level without uricosuria, indeed in the face of uric acid retention.

Discontinuance of salicylate, probenecid and other uricosuric drugs is usually followed by one or 2 days of uric acid retention. This phenomenon, hitherto regarded as "compensatory," would now appear to reflect decline of the plasma drug concentration to the low levels associated with depression of the uric acid clearance. In this connection it should be pointed out that the concentration of drug in the tubular urine is probably more critical than that in plasma, in respect to the reversible action on $C_{uric\ acid}/C_{inulin}$. To be sure, in ordinary circumstances, as in the experiments here recorded, the urinary drug concentration may be presumed to be a function of the plasma drug level but when the two are dissociated—as by concomitant administration of salicylate and sodium bicarbonate—it is found that the determining factor in regulating uric acid clearance is the drug concentration in urine(3,17). The point may be significant in respect to the immediate mechanisms involved.

The uricosuric effects of salicylate, probenecid, phenylbutazone and phenylbutazone derivatives are all ascribable to suppression of tubular reabsorption of uric acid, since clearance studies uniformly show an associated distinct *rise* in C_{urate}/C_{inulin} in each instance(4,6,11,14). Conversely, the clearance data of this paper reveal that the uric acid retention produced by these same drugs in low dosage is uniformly referable to a distinct *fall* in C_{urate}/C_{inulin}. This paradoxical reversibility of effects presumably is related to some characteristic of the tubular transport systems for uric acid, since there is no indication of significant changes in filtered load, *i.e.*, in the filtrability of plasma uric acid(18) or in the glomerular filtration rate. The nature of this characteristic must, for the present, be a matter of speculation. It has already been suggested that a single system for tubular reabsorption of uric acid, presumed to be enzymatic, may be stimulated by low concentrations and inhibited by high concentrations of the same drug(4). Another possibility, not excluded by the evidence at hand, is that 2 more or less distinct systems of tubular transport of uric acid are involved. One of these, of very limited capacity, might be concerned with tubular excretion of uric acid and inhibited by low (and high) drug concentrations; the other, of considerably greater capacity, is clearly responsible for tubular reabsorption of uric acid and evidently is suppressed only by high drug concentrations.

Summary. Salicylate, phenylbutazone, certain phenylbutazone derivatives and probenecid, all of which in high plasma drug concentrations increase the uric acid clearance, are demonstrated by appropriate renal clearance technics to depress the uric acid clearance at low plasma drug concentrations. The significance of this paradoxical reversibility of effects is discussed in relation to controversial aspects of phenylbutazone uricosuria, the phenomenon of "compensatory" uric acid retention after discontinuance of uricosuric agents, and the nature of tubular transport mechanisms for uric acid.

1. Salomé, E. G., *Med. Jahrb.*, 1885, v15, 463.

2. Fauvel, P., *C. r. de l'àcad. d. sciences*, 1907, v144, 932.

3. Yü, T. F., Gutman, A. B., and Sirota, J. H., *Am. J. Med.*, in press.

4. Yü, T. F., Sirota, J. H., and Gutman, A. B., *Clin. Research Proc.*, 1953, v1, 107.

5. Goldring, W., and Chasis, H., *Hypertension and Hypertensive Disease*, The Commonwealth Fund, New York, 1944.

6. Sirota, J. H., Yü, T. F., and Gutman, A. B., *J. Clin. Invest.*, 1952, v31, 692.

7. Buchanan, O. H., Block, W. D., and Christman, A. A., *J. Biol. Chem.*, 1945, v157, 181.

8. Schreiner, G. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 117.

9. Bonsnes, R. W., and Taussky, H. H., *J. Biol. Chem.*, 1945, v158, 581.

10. Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol. and Exp. Therap.*, 1944, v80, 114.

11. Yü, T. F., Sirota, J. H., and Gutman, A. B.,

J. Clin. Invest., 1953, v32, 1121.

12. Brodie, B. B., Yü, T. F., Burns, J. J., Chenkin, T., Paton, B. C., Steele, J. M., and Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 884.

13. Burns, J. J., Rose, R. K., Goodwin, S., Reichenthal, J., Horning, E. C., and Brodie, B. B., *J. Pharmacol. and Exp. Therap.*, 1955, v113, 481.

14. Yü, T. F., Paton, B. C., Chenkin, T., Burns, J. J., Brodie, B. B., and Gutman, A. B., *J. Clin. Invest.*, in press.

15. Wyngaarden, J. B., *J. Clin. Invest.*, 1955, v34, 256.

16. Huffman, E. R., Smyth, C. J., Wilson, Jr., G. M., and Hill, R., *Ann. Rheumatic Dis.*, 1955, v14, 97.

17. Gutman, A. B., Yü, T. F., and Sirota, J. H., *J. Clin. Invest.*, 1955, v34, 711.

18. Yü, T. F., and Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 21.

Received October 13, 1955. P.S.E.B.M., 1955, v90.

Hemolytic Mechanism in Sickle Cell-Hgb C Disease.* (22095)

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Splenic overactivity has been implicated in the hemolysis observed in many of the acquired hemolytic syndromes. The role of the spleen in the red cell destruction characteristic of the hereditary hemolytic anemias, however, has not been clearly defined. The available data suggest basically different mechanisms for the pathogenesis of the increased hemolysis in 2 of the hereditary hemolytic syndromes. In sickle cell anemia (homozygous Hgb S disease) the frequent development of an atrophic spleen, the so-called autosplenectomy, implies that the spleen cannot play an active role in red cell destruction. Furthermore, splenectomy in cases of uncomplicated sickle cell anemia neither leads to hematologic improvement nor results in a prolongation of the lifespan of the erythrocyte. Thus, sickle cell anemia erythrocytes transfused into a splenectomized sickle cell anemia patient still showed a markedly reduced lifespan(1). In hereditary spherocytosis, however, the evidence suggests that the increased destruction of the red cells may be mediated through the presence of a normal spleen, since hereditary spherocytosis erythrocytes transfused into a normal individual lacking a spleen have a normal survival time whereas the same cells given to a normal individual with a spleen demonstrate a sharply reduced lifespan(2). This situation exists irrespective of the pre- or post-splenectomy state of the donor with hereditary spherocytosis, or his hematologic status at the time of study. In view of these considerations, it seemed of some interest to perform similar studies on patients with sickle cell-Hgb C disease. Sickle cell-Hgb C disease is characterized by splenomegaly and signs of hemolysis which appear to vary in degree intermittently during the course of the disease. Because splenomegaly often becomes evident at times of hemolytic crises in sickle cell-Hgb C disease, there is reason to suspect that enlargement of the spleen may lead to accelerated rates of red cell destruction. By defining the part played by the spleen in the hemolysis of this disease, it was hoped that an evaluation of the effects of splenectomy and the possible use of this operation as a therapeutic procedure might thereby be made.

Material and methods. Three patients with the diagnosis of sickle cell-Hgb C disease, confirmed by electrophoretic analysis of their hemoglobin types, were used as donors. All were in a relatively asymptomatic state. The spleens of 2 of the donors were palpable just below the costal margin. From one patient, A. F., two 500 ml aliquots of blood were obtained and transfused into a normal individual with a spleen and into a hematologically normal splenectomized recipient who had

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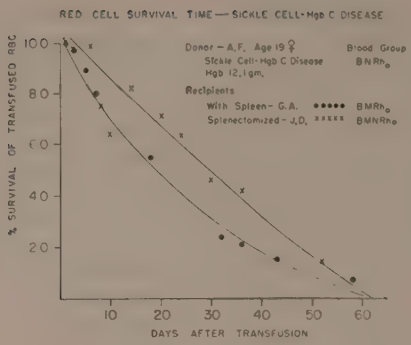

FIG. 1.

been subjected to surgery some years earlier for idiopathic thrombocytopenic purpura. From the second patient, I. F., the younger sister of A. F., 500 ml of blood were removed and transfused into a second splenectomized recipient. The survival time of the erythrocytes was followed by the Ashby technic of differential agglutination in the standard way using dried anti-M serum which gave inagglutinable counts of less than 10,000 cells per cu mm(1). The third donor with sickle cell-Hgb C disease, L. M. (blood type O N Rh_0) was utilized for a combined Ashby and Cr^{51} tagged red cell survival time study. Approximately 500 ml of blood were removed by venesection and three 20 ml aliquots of blood were tagged with 75 microcuries of Cr^{51} by a modification of the method of Weinstein, *et al.*(3). One aliquot of the Cr^{51} tagged cells was auto-transfused into the donor, a second given to a compatible normal recipient (blood type B M Rh_0), the third to a splenectomized normal individual of blood type AB MN Rh_0. The remainder of the blood was transfused into the normal recipient with a spleen who had been given the tagged cells. The disappearance of the Cr^{51} from the peripheral blood, determined at intervals of one or 2 days, was carried out in a well-type scintillation counter and the half-time disappearance (t/2) calculated(3). The Ashby technic was used to follow the survival of the transfused red cells in the normal recipient.

Results and discussion. In the first experiment, performed by the Ashby technic alone, no appreciable difference was noted between the survival times of red cells from the patient with sickle cell-Hgb C disease transfused into the normal and splenectomized individuals. In each instance the red cells disappeared from the recipient's circulation in approximately 50 to 60 days (Fig. 1). Similar data were obtained in the second experiment in which sickle cell-Hgb C disease erythrocytes were transfused into a splenectomized recipient. The red cell lifespan was again approximately 60 days (Fig. 2). Since no normal control was run at the same time, it is impossible to say whether this result represents a prolongation of the erythrocyte lifespan in the splenectomized subject. However, the similarity of the findings in each of

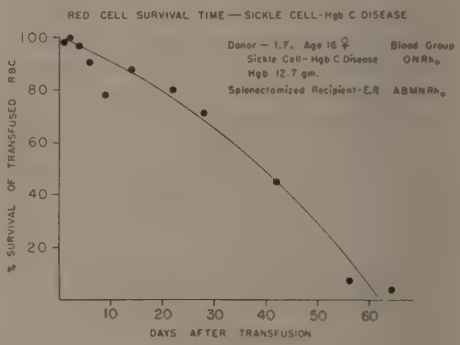

FIG. 2.

the first 2 experiments suggests that any increase in red cell survival time in the splenectomized recipient must have been slight, if at all present. In the third experiment, the half-time of disappearance of Cr^{51} tagged

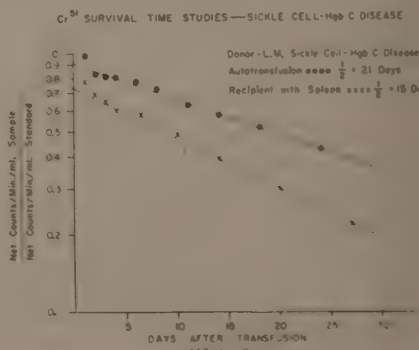

FIG. 3.

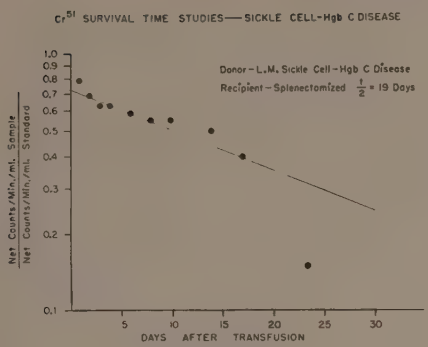

FIG. 4.

cells transfused back into the donor was 21 days, while in a normal individual these cells had a t/2 of 15 days (Fig. 3). The aliquot of labelled sickle cell-Hgb C disease erythrocytes followed in the splenectomized recipient appeared to have a t/2 of approximately 19 days, although a single satisfactory line of disappearance for the Cr^{51} could not be drawn (Fig. 4). The configuration of the curve suggested that 2 or more populations of red cells were present or that the Cr^{51} was being eluted from the pathologic cells at an inconstant rate. Nevertheless, the major proportion of the Cr^{51} disappeared at approximately the same rate in the splenectomized and non-splenectomized recipients. The erythrocyte lifespan by the Ashby technic was, as in the instance of the first 2 donors, approximately 50 days in the normal recipient (Fig. 5).

These observations suggest that the intrinsic defect of sickle cell-Hgb C erythrocytes, like that of sickle cell anemia red cells, is primarily responsible for the shortened survival time of the red cells. As in sickle cell anemia, hemolysis proceeds at approximately the same rate whether a spleen is or is not present. The pathogenesis of the hemolytic mechanism is, therefore, somewhat different from that seen in hereditary spherocytosis where the presence of a normal spleen is required for red cell destruction to take place at an increased rate. These studies do not throw any light on the site of red cell destruction in sickle cell-Hgb C disease. It has been postulated that in sickle cell anemia, by virtue of the abnormal shape of the red cells, plugging of small vessels with resultant stasis and hemolysis takes place. Since this phenomenon may occur in any small vessel suitably situated, the spleen does not selectively trap such cells. In hereditary spherocytosis, the spherical erythrocytes are believed to be selectively trapped in the sinusoids of that organ. It would seem, therefore, that the sickle-shaped erythrocytes in sickle cell-Hgb C disease behave much like the red cells in the related homozygous Hgb S condition.

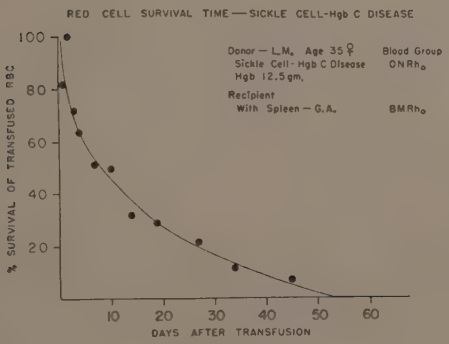

FIG. 5.

In vivo sickling can be correlated with the percentage of the total hemoglobin in the form of Hgb S(4,5). Since the majority of patients with sickle cell anemia have approximately 90% Hgb S(6,7) while those with sickle cell-Hgb C disease have from 45-50% Hgb S(8), it would be expected that under similar conditions hemolysis as a consequence of the mechanical factors mentioned above would take place more readily in sickle cell anemia. Furthermore, since some patients with homozygous Hgb S disease have as little as 60% Hgb S(9) while some with sickle cell-Hgb C disease have over 63% Hgb S(10), some overlap in the severity of the anemia and degree of hemolysis should be encountered if red cell destruction in these sickling diseases is to be explained on the basis of mechanical trapping, stagnation and lysis. Clinical and hematologic data in the 2 sickle cell diseases are consistent with these tenets and support this concept. It would be of extreme interest to try to localize if possible

major sites of stasis and destruction in these sickling disorders. Unfortunately, body scanning following the injection of Cr^{51} tagged cells was not possible, but such studies would undoubtedly provide important relevant information.

Another problem left unanswered by the present investigation is the role of the spleen during periods of clinical crisis. Characteristic of the crisis of sickle cell-Hgb C disease is the progressive development of marked anemia and a rapidly enlarging spleen. The clinical findings are, therefore, in marked contrast to those observed in the usual variety of crisis seen in sickle cell anemia, where the abdominal, muscle and joint pains are not accompanied by any specific hematologic changes nor any increase in the degree of hemolysis(11). Abdominal pain in sickle cell-Hgb C disease is usually localized in the left upper quadrant and can be correlated with the remarkable change in the size of the spleen. It seems more than likely that splenic overactivity, either by virtue of its increasing mass, or as a result of a more specific role in sequestering the abnormally shaped red cells, leads to the development of the severe anemia seen during periods of crisis in sickle cell-Hgb C disease. Furthermore, the number of patients with sickle cell-Hgb C disease who appear to develop the first episode of anemia following trauma to the left upper quadrant area strongly suggests that splenic enlargement may actually be the precipitating factor in the pathogenesis of the crisis, while the anemia is a secondary phenomenon. Unfortunately none of our patients was suffering from this complication at the time of observation, but repeat Cr^{51} auto-survival time studies of the erythrocytes during periods of quiescence and crisis will make it possible to evaluate this problem.

Summary. Red cells from 3 patients with sickle cell-Hgb C disease transfused into normal individuals with and without a spleen, were found to have a survival time of approximately 50 days. The spleen, therefore, does not appear to have a critical role in the hemolysis occurring in this disease. Possible mechanisms for the red cell destruction, based on the per cent of Hgb S present, as well as the role of the spleen during periods of crisis, are discussed.

1. Callender, S. T. E., Nickel, J. F., Moore, C. V., and Powell, E. O., *J. Lab. and Clin. Med.*, 1949, v34, 90.

2. Schrumpf, C. A. A., *Role of the spleen in familial spherocytosis.* Proceed. 3rd Internat. Cong. of Internat. Soc. Hematol., edited by C. V. Moore, Grune & Stratton, New York, 1951, p94.

3. Weinstein, I. W., Spurling C. L., Klein, H., and Necheles, T. F., *Blood,* 1954, v9, 1155.

4. Sherman, I. J., *Bull. Johns Hopkins Hosp.*, 1940, v67, 309.

5. Harris, J. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 197.

6. Wells, I. C., and Itano, H. A., *J. Biol. Chem.*, 1951, v188, 65.

7. Singer, K., Chernoff, A. I., and Singer, L., *Blood,* 1951, v6, 413.

8. Chernoff, A. I., *New Eng. J. Med.*, 1955, v253, 322, 365, 416.

9. Watson, R. J., *The hereditary anemias, Bull. New York Acad. Med.*, 1954, v30, 106.

10. Schneider, R. G., *J. Lab. and Clin. Med.*, 1954, v44, 133.

11. Henderson, A. B., *Am. J. Med. Sci.*, 1951, v221, 628.

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Hyperactivity of Metachloridine against *Plasmodium gallinaceum* in Chicks Maintained on a Purified Diet.* (22096)

D. Jane Taylor and Joseph Greenberg. (Introduced by G. M. Briggs.)

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In a previous report(1), it was shown that sporozoite-induced *Plasmodium gallinaceum* infections behave similarly in chicks fed a purified diet or a commercial stock diet. It was thought, however, that the effect of therapeutic agents might differ in chicks fed different dietary regimens, since several antimalarials are known to be, or are suspected of being, antimetabolites. Commercial stock diets are known to vary in their composition from time to time and conceivably could vary in their content of critical natural components which might inhibit the activity of certain antimalarials. The variability in the content of diet of such natural metabolites might account for the minor variations occasionally encountered in the activity of antimalarials. Alternatively, the presence of antibiotics currently being added to stock feed might have some influence on the results of tests with antimalarials.

We undertook to determine the effect of a purified diet (C-2(1,2)) on the parasitemia produced by *P. gallinaceum* in untreated chicks after the intravenous inoculation of erythrocytic parasites and the effect of such a diet on the antimalarial activity of certain compounds. As will be shown, the use of the purified diet had little or no effect on the antimalarial activity of most of the compounds tested. However, metachloridine (2-metanilamido-5-chloropyrimidine) and sulfadiazine were strikingly more active in chicks fed the purified diet than in those fed the stock diet. The hyperactivity of sulfadiazine might have been the result of the absence of *p*-aminobenzoic acid from the purified diet; however, neither *p*-aminobenzoic nor *m*-aminobenzoic acid had any effect on the antimalarial activity of metachloridine(3), nor, to date, has any natural substance been isolated which can reverse the activity of metachloridine. This drug was placed by Josephson *et al.*(4) in a class of antimalarials characterized as plasmodistatic, acting only during the dividing stages of the parasite. It was predicted that the parasite could be made resistant to members of this class of antimalarials, and Bishop and McConnachie(5) found that resistance to metachloridine can be readily induced. Josephson *et al.*(4) predicted that members of this class would be antimetabolites. The fact that metachloridine was more active in chicks fed the purified diet than in those fed stock diet suggested that the latter diet contained an inhibitor, possibly a substance essential for the parasite. A search for such a substance was undertaken, and the results are reported in this paper.

Methods. New Hampshire chicks from a single source were used throughout the experiments. They were received in the laboratory when one day old, separated into 2 groups, and each group immediately placed on one of the test diets, on which they continued until the end of the experimental period. Weights of the chicks were recorded after they had been on the diets for 7 days and again 4 days later. The drug tests were conducted according to a procedure previously described(6). Week-old chicks were inoculated intravenously with 16×10^6 erythrocytes parasitized with *P. gallinaceum*. Parasite counts (parasitized erythrocytes per 10^4 erythrocytes) were made on the morning of day 4 (day of inoculation is day 0). Drugs were administered orally, twice daily for 4 days, beginning 4 to 5 hours before parasite

* The authors are grateful to the following: the Archer-Daniels-Midland Co. for furnishing the soy grits and hulls; the Soya Products Division of the Glidden Co. for the soy granules; Drs. M. R. Spivey Fox and G. M. Briggs for helpful suggestions; Anne Jowett, G. W. Rusten, and J. Phillips for technical assistance.

TABLE I. Comparison of Antimalarial Activity of 9 Compounds Tested in Chicks Infected with *P. gallinaceum* and Fed Either the C-2 Diet or the Stock Diet.

Compound	Minimum effective dosage* (mg/g body wt) C-2 diet	Stock diet
Quinine	.005	.01
Chloroquine	.001	.002
Atabrine	.005	.01
Chlorguanide	.0005	.002
	.002	.002
Terramycin	.04	.08
Fumagillin	>.04	>.04
Pamaquine	.0003125	<.000625
Sulfadiazine	<.025	.1
	≦.003125	.05
Metachloridine	.000125	.002
	.0000625	.002
	<.0000625	.0005

* Each result derived from one experiment.

inoculation. The stock diet used was Purina Startena; the purified diet was prepared in this laboratory according to published procedures(1,2). Henceforth, the purified diet will be termed the C-2 diet. Variations in the C-2 diet were made by replacing a portion of the Cerelose with the test quantity of the nutrient to be studied. In the experiments designed to test the effect of nutrient variables of the C-2 diet on the antimalarial activity of metachloridine, the drug was given at 5 levels to those chicks given the varied C-2 diet and at 3 levels to those fed the stock diet. In late experiments, the C-2 diet served as the control diet. The corn-soy diet (a complete all-vegetable diet)(7) contained the following constituents per kilo of diet: 575 g of ground, whole, yellow corn; 350 g of soybean meal; 10 g of Cerelose; 5 g of corn oil; 60 g of Briggs chick salts-A(2); 0.02 mg of vit. D_3, 8 mg of riboflavin; and 0.02 mg of vit. B_{12}.

Results. The mean 4-day weight gain of 60 parasitized chicks in 12 series fed stock diet was 20.1 ± 0.6 g; the corresponding 60 chicks fed the C-2 diet gained 18.5 ± 0.9 g. The difference between the means was not significant ($p<0.2$).

The mean 4-day parasitemia of the above 60 chicks fed the stock diet was 7289 ± 251/10^4 erythrocytes; the parasitemia of the corresponding 60 chicks fed the C-2 diet was 6890 ± 225. There was no significant difference between the means ($p<0.3$).

The antimalarial activity of 9 compounds was compared in chicks fed C-2 diet and stock diet (Table I). With 7 of these compounds, the difference in antimalarial activity between the 2 diets was small and probably of doubtful significance. On the other hand, sulfadiazine was at least 16 times as active in chicks fed the C-2 diet as it was in chicks fed stock diet. In preliminary trials metachloridine was about 16 times as active when used to treat chicks fed C-2 diet as it was in chicks fed stock diet.

In repeated trials, it was established that metachloridine was 16 times as active in C-2 diet-fed chicks (Table II). At the suggestion of Dr. George M. Briggs, metachloridine was tested in chicks fed an all-vegetable diet (see Methods), in which the 2 most abundant constituents were ground corn and soybean meal. Metachloridine was no more active with this diet than it was with the stock diet. Ground corn and soybean meal were tested separately as part of the C-2 diet in approximately the concentrations in which they occurred in the corn-soy diet (Table II). When 41% soybean meal (either expeller type or hexane-extracted) was incorporated in the C-2 diet, the hyperactivity of metachloridine associated with the purified diet was almost completely obliterated. Ground corn at 60% of the diet appeared to reverse partially the hyperactivity of metachloridine, but soybean meal appeared to be a richer source of inhibitor and to account for most of the inhibition of metachloridine activity observed in the all-vegetable diet.

The protein of soy at a 33% level was put into the C-2 diet at the expense of Cerelose. In chicks fed this diet, metachloridine was only slightly less active than in chicks fed the unmodified C-2 diet (Table II).

Other fractions of soybean meal were tested in single or duplicate tests (Table III). Metachloridine was as active in chicks fed 4% soybean oil in place of the same amount of corn oil of the C-2 diet, or the residue after alkaline extraction of the meal ("soybean

TABLE II. Effect of Diet on Antimalarial Activity of Metachloridine against *P. gallinaceum* Infected Chicks.

Dosage of metachloridine, mg/g b.i.d. for 4 days	Mean fourth day parasitemia (parasitized rbc/10^4 rbc)				
	Stock	C-2	C-2* 41% soybean oil meal§	C-2† 60% ground corn	C-2‡ 33% soy protein‖
.002	970 ± 106¶				
.001	2164 ± 190¶		1504 ± 297	298 ± 134	369 ± 130
.0005			2582 ± 365	1012 ± 361	402 ± 89
.00025			4008 ± 432	1786 ± 416	949 ± 183
.000125		760 ± 158**	5629 ± 501	3335 ± 490	2034 ± 462
.0000625		2150 ± 280††	6084 ± 277	4449 ± 1040	3917 ± 540
.0000313		3836 ± 350‡‡			
None	7622§§	7268 ± 596	7196 ± 236		

* 5 series, 25 chicks each dose.
† 2 " , 10 " " " .
‡ 3 " , 15 " " " .
§ Expeller type and hexane extracted, data pooled.
‖ Both alpha protein and sodium proteinate, data pooled.
¶ 22 series, 109 chicks.
** 8 " , 40 " .
†† 8 " , 37 " .
‡‡ 10 " , 50 " .
§§ 23 " , 197 " .

granules"), as it was in those fed the regular C-2 diet. The residue remaining after the acid extraction of the meal ("acid-washed grits") appeared to have as much of the inhibitor as the soybean meal. Soybean hulls did not appear to be a rich source of the inhibitor.

Incorporation into the C-2 diet of *p*-aminobenzoic acid (100 mg/kg of diet). 5% brewer's yeast, 5% dried whole beef liver, or twice the concentration of the water-soluble vitamins of the C-2 diet, failed to inhibit the antimalarial activity of metachloridine.

Discussion. Of the 9 antimalarials tested against *P. gallinaceum* in chicks fed the C-2 diet, only sulfadiazine and metachloridine were significantly more active than in chicks fed stock diet. The hyperactivity of sulfadiazine is not surprising, since the C-2 diet has no added *p*-aminobenzoic acid and only a normal amount of folic acid.

TABLE III. Effect of Various Soy Bean Fractions, Incorporated into C-2 Diet, on Antimalarial Activity of Metachloridine against *P. gallinaceum* Infected Chicks.

Fractions of soy beans	Minimum effective dose of metachloridine, mg/g body wt
4% soy bean oil	.000125
" " " "	.000125
41% " " hulls	.0005
" " " granules	.00025
" " " grits (acid washed)	.001

The hyperactivity of metachloridine in chicks fed the purified diet could indicate the absence from this diet of an inhibitor present in the stock diet. Such an inhibitor appears to be present in vegetable products such as soybeans and corn, though the former appears to be a richer source. The inhibitor is not *p*-aminobenzoic acid, nor does it appear to be concentrated in such sources of vitamins as dried yeast or dried liver. It is probably not one of the known water-soluble vitamins for no significant inhibition of metachloridine resulted when the vitamin content of the C-2 diet was doubled.

Neither the fatty fraction nor the protein fraction of soybeans appears to contain significant amounts of the inhibitor. The residue left after the alkaline extraction of soybean meal ("granules") had little or no inhibitor, but the residue after acid extraction ("grits") seemed to have significant amounts of the inhibitor.

The conclusions that can be drawn at this stage of the investigation must be tentative. The data strongly suggest that there is a substance in soybean meal which inhibits the antimalarial activity of metachloridine. This substance is neither fat nor water-soluble protein, nor is it significantly soluble in hexane. It would appear to be alkali soluble, or labile, and acid insoluble. Such a substance could conceivably be a growth factor, a natural product essential for the parasite but not for

the host, and not produced in any abundance by the host. The isolation of such a substance might give some further clues as to what kind of natural products become growth factors for parasites and what kind of chemicals serve as inhibitors of such substances.

Summary. 1. The erythrocytic form of the malarial parasite *Plasmodium gallinaceum* was found to grow at a comparable rate in chicks fed either a purified diet (C-2) or a commercial stock diet. The antimalarial activity of 7 compounds was approximately the same in parasitized chicks fed either of the above diets, while sulfadiazine and metachloridine exhibited a marked increase in effectiveness in those chicks fed the C-2 diet. 2. The hyperactivity of metachloridine in chicks fed the C-2 diet was reversed almost completely when this diet contained 41% soybean meal. Neither the fatty nor protein fractions of soybeans, nor several other dietary nutrients appeared to contain significant amounts of the inhibitor.

1. Greenberg, J., Taylor, D. J., and Trembly, H. L., *Am. J. Hyg.*, 1954, v60, 99.

2. Briggs, G. M., Spivey, M. R., Keresztesy, J. C., and Silverman, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 113.

3. Brackett, S., and Waletsky, E., *J. Parasitol.*, 1946, v32, 325.

4. Josephson, E. S., Taylor, D. J., Greenberg, J., and Coatney, G. R., *J. Infect. Dis.*, 1953, v93, 257.

5. Bishop, A., and McConnachie, E. W., *Parasitol.*, 1953, v42, 277.

6. Coatney, G. R., Cooper, W. C., Eddy, N. B., and Greenberg, J., *Survey of Antimalarial Agents*, Monograph No. 9, Public Health Service Pub. No. 193, U. S. Government Printing Office, Washington, D.C., 1953.

7. Spivey, M. R., Briggs, G. M., and Ortiz, L. D., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 451.

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ERRATUM

Article on Serum Lactic Dehydrogenase . . . by Kuang-Mei Hsieh *et al.*, Vol. 89, p. 627, Tables I, II, and III, second column heading should read "mM substrate oxidized per L serum per hr."

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SECTION MEETINGS

BALTIMORE Camp Detrick, Md.	October 14, 1955
CLEVELAND, O. Western Reserve University	November 21, 1955
DISTRICT OF COLUMBIA George Washington University	December 1, 1955
ILLINOIS University of Illinois Med. School	November 1, 1955
IOWA State University of Iowa	October 25, 1955
MINNESOTA Mayo Foundation	November 11, 1955
WESTERN NEW YORK Cornell University	November 12, 1955
SOUTHWESTERN University of Oklahoma	November 18-19, 1955

Grouping and Occurrence of RI (Prototype RI-67) Viruses.* (22097)

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Viruses of the recently discovered RI family(1-4) which includes agents designated APC(5-8) or ARD(9) may cause undifferentiated acute respiratory disease (ARD)(10), non-*Streptococcal* exudative pharyngitis(11), primary atypical pneumonia unassociated with cold agglutinins(11), pharyngo-conjunctival fever(5-7), mesenteric lymphadenitis(12) or inapparent infections(2,4) in man. These viruses are heterogeneous antigenically(1,6,7,13) comprising a group of distinct serotypes(6,7) which are readily differentiable by the serum neutralization technic; however, they also elaborate a common group-specific "soluble" complement-fixing antigen which is readily separable from the virus(7,14,15) and which appears to be of little if any significance in immunity to RI disease(2,4). The seemingly ubiquitous distribution of RI agents not only in patients who are sick but also in latent or "masked" form in human adenoid or tonsil tissue(6,7, 16) poses the problem of determining which

* The majority of these observations were presented at 39th annual meeting of Am. Assn. of Immunologists, San Francisco, Apr. 11-15, 1955.

serotypes are the more important in causing respiratory illness and which are less frequently associated with human disease. The present paper summarizes the results of a study to determine the occurrence of the RI virus types in epidemics of acute respiratory illness among newly recruited soldiers in training. The antigenic grouping of the RI viruses based on tests with serum pairs from patients is also presented. Additionally, information concerning the serological response to homologous and heterologous virus serotypes in natural and experimental infections in man is summarized.

Materials and methods. Clinical. The patients with acute respiratory illness from whom blood samples and throat washings were obtained were U.S. Army personnel stationed at Fort Dix, N. J., Fort Leonard Wood, Mo. or Fort Ord, Calif.† during the winter of 1953, 1954 or 1955 when there was a high prevalence of infection with the RI viruses. Other articles(1-4,17) describe these epidemics. Sera were also available from volunteers who had been inoculated experimentally with the RI-67 strain of virus (18). *Laboratory. Virus isolation.* The methods employed for recovery of viruses from patients' throat washings, for propagating these agents, and for preparing complement-fixing (CF) antigens in HeLa(19) cell tissue cultures‡ were described earlier(1). *Antigenic typing of strains.* The recovered viruses were typed by the serum neutralization procedure(1) in HeLa cell cultures employing specific rabbit antisera according to the classification system§ of Rowe *et al.*(7). Seed virus stocks used to immunize rabbits or employed in the neutralization tests were prepared in HeLa cell tissue cultures. Neither antiviral antisera nor antisera prepared in rabbits against uninfected HeLa culture material had a demonstrable adverse effect upon the cells in the tissue cultures when tested at a dilution of 1:4. *Serology.* Serum neutralization and CF tests with the RI viruses and patients' sera were performed according to procedures outlined(1) for use with the RI-67 strain. Serum titers were expressed as the greatest initial dilution of patient's serum which caused complete or nearly complete suppression of viral growth (neutralization) or of fixation of complement. In the neutralization tests presented in Table I, one serum pair was tested with all 11 viruses on one day. The same seed virus stocks were employed throughout.

Results. Determination of viruses important in recruit disease and antigenic grouping of strains. The acute and convalescent sera from 20 soldiers with undifferentiated acute respiratory disease (ARD) or primary atypical pneumonia of RI etiology as proved by results of CF tests were examined for increase in neutralizing antibody against 11 RI virus strains which had been isolated from patients suffering with these illnesses and also against types 1, 2, 5 and 6 of the latent viruses recovered by Rowe, Huebner *et al.* from human adenoid or tonsil tissue. The findings, presented in interpretive form in Table I, express in terms of numerical designations the relative fold-increase in serum titer of each serum pair against each virus. In the tests, each convalescent serum showed a 32-fold or greater rise in titer for at least 1 virus of the group. It is seen in the table that by far the greatest increase in antibody was against the viruses which were recovered from soldiers who were ill with respiratory disease. By contrast, the serological response to the types 1, 2, 5 and 6 latent viruses was notably weak or absent. Thus, these particular latent agents did not appear to be of etiological significance in the illnesses among the military population in this study. Further, they were of little importance in the studies of others (9) who have examined military populations.

† We are indebted to Lt. Col. T. O. Berge for supplying the 5 strains of virus recovered from patients at Fort Ord, and to Lt. Col. Berge, Lt. Col. H. E. Shuey, Col. L. G. Thomas, Dr. E. H. Lennette, and Capt. J. J. McCue for the serum specimens from this location.

‡ HeLa cell cultures and tissue culture fluids employed were obtained from Microbiological Associates, Inc., Bethesda, Md.

§ Prototype 1, 2, 3 (strain GB), 5 and 6 viruses were obtained from Dr. R. J. Huebner and type 7 virus, recently described by Berge *et al.*(20) was supplied by Lt. Col. T. O. Berge.

TABLE I. Grouping of RI Virus Strains Based on Increase in Neutralizing Antibody in Paired Sera from Patients with Respiratory Illness. Interpretive summary.

Sera from RI patient		Increase in neutralizing antibody against RI strains														
		Viruses recovered from soldiers ill with respiratory disease											Latent viruses			
		Group A–Type 4		Group B–Type 7					Group C–Type 3				T1†	T2	T5	T6
No.	Fort*	67	C-1	4-202	4-239	4-204	4-218	3-83	3-110	C-2	3-131	GB	AD-71	AD-6	AD-75	TD-99
4-385	Ord	5	5	2	2	2	2	2	2	0	0	0	0	0		
465	"	5	5	0	3	1	1	3	0	0	0	0	2	0		
3-33	L. Wood	5	5	1	2	2	0	2	0	2	2	2	0	0		
63	"	5	5	0	0	0	0	0	0	3	0	0	0	0		
4-245	Dix	5	5	2	0	0	0	0	1	1	2	1	1	0	0	0
306	L. Wood	5	5	1	0	1	3	0	0	0	0	0	2	1	0	3
354	Ord	5	5	0	0	0	0	0	0	0	0	0	2	0		
4-305	L. Wood	1	1	5	5	3	1	1	0	0	0	1	0	0		
316	"	0	2	5	3	5	3	3	0	0	0	0	0	0		
472	Ord	1	0	1	5	0	3	5	0	0	1	0	0	0		
488	"	1	0	0	5	0	1	3	0	0	0	0	0	0	0	0
302	L. Wood	2	1	5	3	3	3	3	0	0	0	0	0	0		
202	Dix	0	0	5	3	3	1	1	0	0	0	0	0	0	0	0
239	"	0	0	4	5	3	3	3	2	0	2	0	0	0		
4-233	Dix	5	5	3	5	3	5	3	5	4	5	3	0	0	0	0
3-57	L. Wood	3	3	1	5	1	2	3	2	0	2	2	0	0		
26	"	5	5	0	0	0	0	0	3	5	3	5	0	0		
4-237	Dix	0	0	4	4	2	4	4	4	5	4	5	0	0		
353	Ord	0	0	5	3	3	3	4	2	2	4	4	0	0		
3-134	L. Wood	0	0	1	5	1	0	3	1	2	0	2	0	0	0	0

A designation of 5 indicates the maximum increase in titer obtained with the given pair of sera. A 4 indicates a rise in titer half as great as the maximum; 3, ¼ as great; 2, ⅛ as much progressing consecutively to no rise which is represented by 0.

* Fort Ord, Calif.; Fort Leonard Wood, Mo.; Fort Dix, N. J.

† T = type.

TABLE II. Interpretive Summary of CF Test Results in Paired Sera from Patients with RI Infection. (The system for designation is explained in Table I.)

Sera from RI patient No.	Increase in complement-fixing antibody against RI strains											
	Group A–Type 4		Group B–Type 7				Group C–Type 3		T1*	T2	T5	T6
	67	C-1	4-202	4-239	4-204	4-218	3-110	GB	AD-71	AD-6	AD-75	TD-99
4-245	4	5	5	5	5	5	5	5	5	5	5	5
306	5	5	5	5	5	5	5	5	5	5	5	5
354	5	5			5	5			4	5		
4-188	4	4	4	5	5	5	4	4	4	4	5	5
202	5	5	5	5	5	5	5	5	4	4	4	4
239	3	3	4	4	5	4	5	5	2	2	0	2
4-233	5	5	5	5	5	5	5	5	5	4	5	4
3-57	4	5	4	4	4	4	4	3	3	2	5	4
4-237	5	5	5	5	5	5	5	5	5	4	5	5
353	3	4	4	5	5	4	5	5	3	3	4	3
134	5	5	4	5	4	5	5	5	5	5	5	5

* T = type.

Table I also showed that the viruses recovered from patients with respiratory disease were antigenically heterogeneous. Nevertheless, the strains could be divided into 3 separate groups, designated A, B or C in Table I, based on the reaction with the paired sera from the patients. The first 7 pairs of sera listed showed a sharp rise in titer (designated 5) against the group A strains but little or none against the B or C agents. Conversely, the next 7 pairs of sera listed in Table I usually presented a marked increase in antibody against the B but not against the A or C viruses. The last 6 pairs of sera gave strong rises against the C viruses, and also against either the A or B strains or both. Subsequent typing of these agents with monotypic rabbit antisera demonstrated all of the A strains to belong to Rowe *et al.'s* type 4 while all of the B and C strains belonged to types 7 and 3, respectively. Thus, there was complete agreement as regards grouping of the strains either when the viruses were typed with paired sera from cases or with monotypic rabbit antisera. It is to be noted additionally in Table I that there was greater heterogeneity within the B and C groups than among the A strains. Thus, the 2 A viruses reacted to the same extent with all of the first 7 pairs of sera while, by contrast, the B and C strains varied markedly in their reaction to the second and third groups of sera, respectively. This indicated that there was minor antigenic heterogeneity within these types which could be detected with human sera but which was not revealed in the tests with rabbit antisera.

Complement-fixation findings. The results of CF tests performed with a portion of the paired human sera shown in Table I and representative viruses are presented in Table II. The CF findings were strikingly group-specific in contrast to the highly type-specific reactions obtained in the neutralization test. Serum pair 4-239 was the notable exception in that it showed a weak reaction with the type 1, 2, 5 and 6 latent virus antigens even though the reaction with types 4, 7 and 3 was strong. This finding suggested that there may be a small degree of specificity demon-

TABLE III. Distribution of RI Virus Types among Strains Recovered from Military Patients.

Installation	Time of collection of specimens	No. of strains of type: 3	4	7	Total No. of strains
Fort Dix, N. J.	Jan. 11-13, '54			11	11
	" 31, '55	2	5	2	9
Leonard Wood, Mo.	" 4-9, '53	2	1	2	5
Ord, Calif.	Winter, '54	1	2	2	5*
	Total	5	8	17	30

* Viruses recovered by Lt. Col. T. Berge, Fort Baker, Calif.

TABLE IV. Distribution Neutralizing Antibody Increases for RI Viruses among 25 Patients with RI Infection.

Fold-increase in neutralizing antibody titer	No. of persons showing antibody increase against RI strains Type 3 (3-110)	Type 4 (RI-67)	Type 7 (4-202)
0	15	4	7
2	2	2	3
4	1	3	3
8	2	6	4
16	2	0	
32		9	3
64	1		
128	2	1	4
256			1
No. positive (4-fold or > increase)	8	19	15

strable among the "soluble" antigens of these viruses when tested with sera from certain patients.

Distribution of virus types among the patients. Thirty RI virus strains recovered from patients with respiratory disease at Fort Dix, Fort Leonard Wood or Fort Ord were typed and the distribution of the various serotypes in these military camps is shown in Table III. It is seen that types 3, 4 and 7 only were represented and that all 3 types were present in all 3 camps in spite of their wide geographic separation. The 1954 outbreak at Fort Dix was of special interest in that it was largely if not entirely due to type 7 virus alone(17). To obtain further information about the occurrence of these agents, the paired sera from 25 patients with RI infection selected from the same populations in the same epidemics from which the viruses in Table III were recovered were tested for increase in neutralizing antibody against type 3, 4 and 7 viruses. Table IV, which summarizes the increases in antibody against the strains, showed that 8 patients developed a significant rise in titer against type 3 virus, 19 against type 4 and 15 against type 7. Although one cannot always be confident of the infecting type when the serological response alone is measured (see Table V,) the data are sufficiently clear to permit the conclusion that types 4 and 7 virus were the most common in these particular military outbreaks.

Homotypic and heterotypic response to infection. The findings in tests to determine the type-specificity of the serological response to natural infection among patients in whom the infecting type was definitively established by recovery of virus are summarized in Table V. The increases in neutralizing antibody were predominantly homotypic although the heterologous response to type 3 or 4 virus in the last three cases in the table was as large or larger than that against the type 7 strains which were recovered from the throat washings. Patient 4-246, in whom the heterologous response was 16 times greater than the homologous, provided the most outstanding example of this experience. The serologic findings and the identity of the isolate were determined on several occasions. The possibility cannot be excluded, however, that the patient may have been infected with both type 3 and 7 virus within the time period for collection of the serum specimens. Similar tests to determine the heterotypic response in patients infected experimentally with the RI-67 strain of type 4 virus by several routes are summarized in Table VI. It is noteworthy that the neutralizing antibody response in these individuals was entirely type-specific and there was no rise to either heterologous type. It is of further interest that the artificial infection gave no heterotypic booster effect even in the patients who had antibody against the heterologous virus prior to inoculation with the RI-67 agent.

Discussion. It is evident that the RI viruses recovered from patients with respiratory illness exhibited marked antigenic heterogeneity when tested by the neutralization method with paired sera from cases and that these strains were clearly differentiated into three serological groups. These groups, which correspond to types 3, 4 and 7 as determined by tests with monotypic rabbit antisera(7), showed further minor antigenic heterogeneity within types 3 and 7, the significance of which remains to be determined.

All the viruses recovered from patients belonged to type 3, 4 or 7 and the antibody response in the persons with respiratory disease was against these 3 types primarily. There was little or no antibody produced against the type 1, 2, 5 or 6 latent viruses(7,16) recov-

TABLE V. Homotypic and Heterotypic Neutralizing Antibody Response in Patients with RI Infection from Whom Virus Was Recovered.

Case No.	Virus isolate type	Serum specimen	Antibody titer: RI group CF	Neutralizing: Type 3	Neutralizing: Type 4	Neutralizing: Type 7
55- 11	3	A*	0†	0†	0	0
		C	80	512	0	8
55- 33	3	A	0	0	2	0
		C	5	128	2	0
55- 9	4	A	0	0	0	0
		C	160	8	32	0
55- 8	4	A	0	2	0	0
		C	20	8	32	2
4-229	7	A	0	0	0	0
		C	80	0	8	512
4-239	7	A	0	ND‡	0	2
		C	20		0	512
4-202	7	A	0	ND	0	0
		C	160		0	32
4-207	7	A	0	ND	0	0
		C	160		8	32
55- 6	7	A	0	0	0	0
		C	20	0	8	32
4-204	7	A	5	ND	0	0
		C	80		2	8
4-215	7	A	0	ND	0	0
		C	40		2	8
4-201	7	A	0	0	2	0
		C	20	0	2	8
4-218	7	A	0	ND	0	0
		C	80		8	8
4-233	7	A	0	ND	0	0
		C	80		128	32
4-246	7	A	0	0	0	0
		C	10	128	0	8

* A = acute, and C = convalescent specimen collected 3 or 4 wk later.
† 0 titer in CF test = less than 1:5 and in neutralization test = less than 1:2, the lowest serum dilutions tested.
‡ ND = Tests not done.

ered from human adenoid or tonsil tissue in tissue culture and these latter agents did not appear to be of etiological significance in any of the cases studied. All 3 serotypes 3, 4 or 7 were present in troops in each of the 3 camps and it is to be noted that the personnel in these installations were recruited from the eastern (Fort Dix), central (Fort Leonard Wood) and western (Fort Ord) regions of the United States. Types 4 and 7 virus appeared most often among the soldiers while type 3 was less prevalent. One may speculate that the less frequent occurrence of type 3 might have been related to immunity resulting from prior infection with this agent which is known to cause epidemics in children(5,8).

Patients infected naturally with type 3, 4 or 7 virus usually exhibited antibody increase of low order against at least one other type even though antibody against the heterotype was absent from the acute phase specimen. By contrast, volunteers given type 4 virus (RI-67) by the respiratory route or parenterally gave a homotypic response only even in persons who had antibody initially against another type. The reason for this difference was not evident although it may be related to the fact that the infection in the volunteers was inapparent or associated with minor illness(18).

The present findings are of importance in the laboratory diagnosis of RI infections and in any consideration of immunologic control of the disease. For routine laboratory diag-

TABLE VI. Homotypic and Heterotypic Neutralizing Antibody Response in Volunteers* Given RI-67 Virus.

Patient No.	Age and sex	Virus inoc. route	Serum specimen†	Antibody titer: CF‡ RI group	Neutralizing‡ Type 3	Neutralizing‡ Type 4	Neutralizing‡ Type 7
1	71 ♂	Nebulizer	Pre	0	2	0	8
			Post	0	2	32	8
5	29 ♂	Nebulizer & intraderm.	Pre	0	8	0	0
			Post	160	8	128	0
6	53 ♂	*Idem*	Pre	0	2	8	0
			Post	5	2	128	0
7	54 ♂	"	Pre	0	0	8	0
			Post	40	0	512	0
8	43 ♀	"	Pre	0	0	0	0
			Post	160	0	512	0
9	63 ♂	Intranasal & intraderm.	Pre	0	0	2	0
			Post	10	0	32	0
10	21 ♂	Intramusc.	Pre	0	0	0	2
			Post	80	0	512	2
11	55 ♀	Intramusc. & intraderm.	Pre	0	2	0	0
			Post	40	2	512	0
12	43 ♀	Intramusc.	Pre	0	2	2	0
			Post	20	2	128	0
13	53 ♂	Intramusc. & in'tumor	Pre	0	2	0	0
			Post	0	2	128	2

* Details of the volunteer experiments will be reported elsewhere(18).

† Pre-inoculation specimen collected immediately prior to administration of virus and post-inoculation sample taken 8 to 45 days later.

‡ 0 titer = less than 1:2 in the neutralization test; less than 1:2 in the pre-inoculation CF samples and less than 1:5 in the post-inoculation specimen.

nosis of RI infections, the CF test is the method of choice since a single antigen suffices to detect antibody increase in all cases. Where additional information of the serotype is important, the specific neutralization test with types 3, 4 and 7 virus should be employed or the virus itself should be recovered and typed. As regards immunization of newly recruited soldiers, one would certainly wish to induce resistance against types 3, 4 and 7 viruses.

Summary. Viruses of the RI family which caused acute respiratory illness among soldiers at Fort Dix, N. J., Fort Leonard Wood, Mo., and Fort Ord, Calif. during the last few years were separated into 3 groups based on neutralization tests with paired sera from cases. These 3 groups of agents corresponded to serotypes 3, 4 and 7 of Rowe *et al.* determined by tests with monotypic rabbit antisera. Latent virus types 1, 2, 5 and 6 which were recovered originally from tonsil or adenoid tissues by others did not appear to be of etiologic importance in the military illnesses. The CF test results, in contrast to the neutralization findings, were markedly group-specific. The importance of strain heterogeneity in relation to diagnosis and immunity is discussed.

1. Hilleman, M. R., and Werner, J. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 183.

2. Hilleman, M. R., Werner, J. H., Dascomb, H. E., and Butler, R. L., *Am. J. Pub. Health,* 1955, v45, 203.

3. Hilleman M. R., Werner, J. H., Adair, C. V., and Dreisbach, A. R., *Am. J. Hyg.,* 1955, v61, 163.

4. Hilleman, M. R., Werner, J. H., Dascomb, H. E., Butler, R. L., and Stewart, M. T., *ibid.,* 1955, v62, 29.

5. Parrott, R. H., Rowe, W. P., Huebner, R. J., Bernton, H. W., and McCullough, N. M., *N.E.J. Med.,* 1954, v251, 1087.

6. Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H., and Bell, J. A., *ibid.,* 1954, v251, 1077.

7. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., and Parrott, R. H., *Am. J. Hyg.,* 1955, v61, 197.

8. Bell, J. A., Rowe, W. P., Engler, J. I., Parrott, R. H., and Huebner, R. J., *J.A.M.A.,* 1955, v157, 1083.

9. Ginsberg, H. S., Badger, G. F., Dingle, J. H.,

Jordan, W. S., Jr., and Katz, S., *J. Clin. Invest.*, 1955, v34, 820.

10. The Commission on Acute Respiratory Diseases, *Am. J. Pub. Health*, 1946, v36, 439.

11. ———, *Medicine*, 1947, v26, 441.

12. Kjellen, L., *Arch. f. d. ges. Virusforsh.*, 1955, v6, 45.

13. Hilleman, M. R., Werner, J. H., and Stewart, M. T., *Fed. Proc.*, 1955, v14, 465.

14. Hilleman, M. R., Tousimis, A. J., and Werner, J. H., *Bact. Proc.*, 1955, 61.

15. ———, PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 587.

16. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H., and Ward, T. G., *ibid.*, 1953, v84, 570.

17. Dascomb, H. E., and Hilleman, M. R., *Am. J. Med.*, in press.

18. Southam, C. M., Hilleman, M. R., and Werner, J. H., *J. Lab. and Clin. Med.*, in press.

19. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

20. Berge, T. O., England, B., Mauris, C., Shuey, H. E., and Lennette, E. H., to be published.

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Multiplication of Virulent Poliovirus in Capuchin Monkey Kidney Cultures Without Microscopically Observed Cytopathogenicity.* (22098)

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Previous work from this laboratory demonstrated that the South American ringtail monkey (*Cebus capucina*) has a limited susceptibility to poliovirus *in vivo*(1). Recent experiments showed that the Type 2 Y-SK strain, highly cytopathic for rhesus kidney cultures, was without demonstrable effect in capuchin cultures(2). The present report extends the earlier findings in demonstrating that Type 1 human strains can multiply in epithelial cultures of capuchin kidney, even though no obvious cytopathic changes could be observed. In the course of these experiments, material became available to determine whether capuchin cultures, which supported virus growth with no apparent cell destruction, could act as a selective medium for the propagation of non-neurotropic variants of poliovirus‡(3-8).

Materials and methods. Four Type 1, four Type 2 and three Type 3 strains were employed, both as stool suspensions of patients ill with poliomyelitis and as the first passage material from infected rhesus kidney cultures (in which they were all markedly cytopathogenic). The procedures in use in this laboratory for growing cultures, for isolation and titration of viruses have been reviewed recently(9). In order to test for survival of virus under the conditions of the experiments, control tubes were inoculated and incubated together with the capuchin cultures. The control tubes contained medium alone (without cells) or rhesus cells that had been killed by freezing. Normal controls of uninoculated capuchin cultures were also included. For each test 0.1 ml of fluid was inoculated into a series of 8 to 10 monolayer cultures of capuchin kidney cells and 0.9 ml of lactalbumin hydrolysate medium(9) was added. The cultures were allowed to incubate for various times; at the end of each incubation period, the fluids were harvested, and fresh medium was added if the incubation was to be continued. Titrations were carried out in rhesus kidney cultures, using 8 tubes per dilution, since the capuchin cultures showed no apparent cytopathic alterations. The plaque technic was carried out according to the method of Dulbecco and Vogt(10). Tests for neurotropic properties of the Type 1 strains were carried out by inoculating the first or second passage capuchin culture fluid into

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TABLE I. Passage of Type 1 Poliovirus in Cultures of Capuchin Monkey Kidney.

Strain	Passage in capuchin cultures	Conc. of TC fluid inoc.	Incubation period (days)	TCD_{50} titer in rhesus cultures (per ml)
Easton -15	1	10^0	14	5.0*
	2	"	14	4.1*
	3	10^{-1}	7	>5.0
		10^{-2}	7	4.6
		10^{-3}	7	2.0
		"	11	>4.0
	4	10^{-3}†	7	1.7
		" †	11	3.1
		" †	18	4.0
	5	10^0	2	4.6
NE-23	1	10^0	14	4.3*
	2	10^{-1}	7	5.8
		10^{-3}	7	>4.0
		"	11	>5.0
	3	10^{-4}†	7	3.0
		" †	11	4.3
		" †	18	4.4
	4	10^0	2	4.7
NE-34	1	10^0	14	4.0*
	2	10^{-1}	7	5.4
		10^{-3}	7	>4.0
		"	11	>5.0
	3	10^{-4}†	7	2.8
		" †	11	2.8
		" †	18	3.6
	4	10^0	2	5.1

* Virus could not be recovered from tubes containing medium alone or rhesus cells killed by freezing, which were inoculated with the same amount of virus and incubated for the same period of time as these capuchin cultures.

† These dilutions were made of the fluids harvested from the cultures of the previous passage which had been inoculated with virus diluted a thousandfold.

rhesus monkeys, 2 intracerebrally (0.5 ml) and 2 intraspinally (0.1 ml). The 4th or 5th passage material was tested in 6 monkeys for each of the strains.

Results. The Type 1 strains grew readily in trypsinized capuchin kidney cultures, although no cytopathic changes could be observed in the microscope. The infected cultures looked exactly like the uninoculated controls. No evidence could be obtained to show that Type 2 and 3 strains multiplied under the same circumstances. The results of the titrations and the number of passages for three Type 1 strains are given in Table I. There are 3 lines of evidence to show that multiplication of virus occurred:

1. All the passages were made by the addition of 0.1 ml of virus or harvested fluids into 0.9 ml of medium in the culture tubes. Successive dilutions for the 5 passages of strain E-15 were 10^1, 10^1, 10^4, 10^4, and 10^1, making the final dilution of the original material 10^{11}. The titer of virus was never greater than 10^5 TCD_{50}/ml, and since virus was present in the final fluid at a titer of $10^{4.6}$, virus multiplication must have occurred. This is supported by the failure to obtain virus from the control tubes (no living cells present) incubated during the first two passages together with the capuchin cultures.

Similar considerations apply to strains NE-23 and NE-34; these also showed the presence of virus at relatively high levels in the final culture fluids, which represented a dilution of 10^{11} of the original fluids.

2. Multiplication was shown as well by the increase of virus during a single passage. When capuchin cultures were inoculated with less than 10 TCD_{50} of strain E-15 in its second passage, fluids with a titer of greater than $10^{4.0}$ were obtained, indicating that at least a thousandfold increase in virus concentration had occurred. This type of virus increase was obtained several times and also with the other Type 1 strains.

3. Virus propagation was also demonstrated by titrations of fluids taken at different times from the culture tubes. With passage 3 of the E-15 strain, fluids harvested from the cultures inoculated with 10 TCD_{50} yielded a titer of $10^{2.0}$ at the end of 7 days and one greater than $10^{4.0}$ at the end of 11 days of incubation. This increase of virus concentration with time is shown also in passage 4: the titers of the harvested fluids were $10^{1.7}$, $10^{3.1}$, and $10^{4.0}$ at the end of 7, 11, and 18 days, respectively. Similar results were obtained with the other two strains.

To determine whether all the capuchin cells in the culture produced virus but without cellular degeneration or whether only a few cells were involved in virus propagation, the following experiment was done.§ Capuchin monkey kidney cells were grown in petri

§ These experiments were carried out by one of us (A.S.K.) at the California Institute of Technology through the hospitality of Drs. Renato Dulbecco and Marguerite Vogt.

TABLE II. Number of Infected Cells in Capuchin Monkey Kidney Cultures as Determined by the Plaque Technic.

Day	No. of cells in inoculum (× 100,000)	No. of plaques	% of cells infected
1	2	105	.05
2	1.1	31	.03
3	1.8	27	.015
6	.1	21	.21
7	1.7	39	.023

plates according to the technic of Dulbecco and Vogt(10). These monolayer cultures were inoculated with 0.1 ml of Easton-15, 4th passage capuchin virus; the inoculum contained 2 x 10^5 plaque-forming particles (as determined by titration on rhesus plates). Nutrient medium was added, and at the intervals shown in Table II, the medium was removed, the plates washed several times with buffered saline, and the monolayer of cells suspended by means of 0.02% versene. The cells were sedimented and resuspended in 1 ml of nutrient medium; 0.1 ml of this cell suspension was seeded onto petri plate cultures of rhesus monkey kidney cells. After a period of 30 minutes to allow for adsorption of virus, an agar overlay was added. The plaques were counted at the end of 4 days.

The results shown in Table II demonstrate that in a population of 10^5 cells only an average of 60 cells was infected. This experiment shows that the absence of obvious cytopathic changes in the capuchin monkey kidney cultures was due to the fact that only a small proportion of cells was involved in the production of virus. The degeneration of so few cells in the culture would be very difficult to detect microscopically. The propagation of certain polioviruses in tissue cultures in the absence of observed cytopathic changes has previously been recorded(12-14), and it is likely that the present explanation of only a small proportion of cells supporting virus propagation, underlies the earlier observations.

Neurotropic properties. After 4 to 5 passages in capuchin cultures the 3 Type 1 strains were compared for the ability to grow in the central nervous system of rhesus monkeys. Attenuated strains of poliovirus have been characterized by their restricted growth in the monkey nervous system, limited chiefly to the lumbar part of the spinal cord(11). Thus an intracerebral inoculation of attenuated virus would be expected to produce disease rarely—only if the virus in some manner found its way to the lumbar region. As a corollary, intraspinal inoculation would be expected to produce paralysis of the legs, if at all. In the present experiments the viruses

TABLE III. Neurotropism of Poliovirus in Rhesus Monkeys, after Propagation without Microscopically Observed Cytopathogenicity in Capuchin Cultures.

Strain	No. of passages in TC	Route	Doses* (× 1000)	Result,† day of paralysis
E-15	2	Intracerebral	8	7 (L); inc.
		Intraspinal	1.6	4 (L→A); remained well
	5	Intracerebral	20	7 (A + L); 10 (L); 14 (A + L)
		Intraspinal	4	4 (L→A); 4 (L→A); 4 (L)
NE-23	1	Intracerebral	5	8 (A + L); 8 (A)
		Intraspinal	1	4 (L); 4 (L)
	4	Intracerebral	25	14 (T + L); 14 (T + L); inc.
		Intraspinal	5	2 (L→A); 3 (L→A); 3 (L→A)
NE-34	1	Intracerebral	0.8	5 (L); inc.
		Intraspinal	0.2	4 (L); inc.
	4	Intracerebral	63	8 (A + L); 11 (L); 14 (L)
		Intraspinal	12.6	3 (L→A); 4 (L→A); 4(L)

* Doses in inoculum determined as $TCID_{50}$ for rhesus kidney cultures on the day monkeys were inoculated.

† L = leg paralysis; A = arm paralysis; T = tremors; inc. = incomplete test due to nonspecific death of monkey. 4 (L→A) indicates that paralysis first occurred on the 4th day, the legs being involved. Subsequently the arms (A) became involved.

propagated in capuchin kidney cells remained virulent, in the doses inoculated, after 4 or 5 serial tissue culture passages. As shown in Table III, paralytic disease was produced after intracerebral inoculation. In addition, the affinity of the virus for the CNS was demonstrated after intraspinal inoculation. When the virus was injected into the lumbar area, it multiplied there and frequently ascended the cord; this manifested itself by leg paralysis followed by arm paralysis.

Summary. Cultures of trypsinized kidneys of South American capuchin monkeys (*Cebus capucina*) supported the multiplication of Type 1 polioviruses, even though no cytopathic changes were observed by microscopic examination of the cultures. The failure to observe cytologic changes was found to be due to the small proportion of cells in the culture capable of supporting the growth of virus. After 4 to 5 tissue culture passages the viruses remained virulent for the monkey brain and spinal cord. No evidence was obtained for the propagation of Type 2 and 3 strains in the capuchin cultures.

1. Melnick, J. L., and Paul, J. R., *J. Exp. Med.*, 1943, v78, 273.
2. Kaplan, A. S., *Virology*, 1955, v1, 377.
3. Theiler, M., *Medicine*, 1941, v20, 443.
4. Koprowski, H., Jervis, G. A., and Norton, T. W., *Am. J. Hyg.*, 1952, v55, 108.
5. Enders, J. F., Weller, T. H., and Robbins, F. C., *Fed. Proc.*, 1952, v11, 467.
6. Melnick, J. L., *ibid.*, 1954, v13, 626.
7. Sabin, A. B. Hennessen, W. A., and Winsser, J., *J. Exp. Med.*, 1954, v99, 551.
8. Li, C. P., and Schaeffer, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 148.
9. Melnick, J. L., *Ann. N. Y. Acad. Sci.*, 1955, v61, 754.
10. Dulbecco, R., and Vogt, M., *J. Exp. Med.*, 1954, v99, 167.
11. Sabin, A. B., *ibid.*, 1955, v61, 924.
12. Ledinko, N., Melnick, J. L., and Riordan, J. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 83.
13. Youngner, J. S., Ward, E. N., and Salk, J. E., *Am. J. Hyg.*, 1952, v55, 291.
14. Sabin, A. B., *Science*, 1954, v120, 357.

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Measure of Irritation by Quantitative Determination of Extravasated Dye.* (22099)

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Intravenously administered colloidal dyes have been observed to concentrate at sites of inflammation(1-5). This phenomenon has been the basis for tests to measure the intensity of irritation(6-10). In such tests, visual estimations of the quantity of dye at irritated sites were graded by various systems, either direct or by comparison to standard color charts. The results of visual observations in this manner are influenced by subjective bias and are not easily reproducible.

This report describes a method for the quantitative removal of accumulated Evans blue dye from irritated tissue and its measurement spectrophotometrically. This should be a reliable index of the degree of irritation, provided the amount of extravasated dye is proportional to the extent of irritation. A modification of the method of Caster, Simon, and Armstrong(11,12) for the measurement of plasma volume was used for extracting the dye from tissue.

Methods and materials. White rats, (Sprague-Dawley), 115-130 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital. The skin on the posterior side of the hind legs was slit to expose the gastrocnemius muscles and 0.03 cc of the test material was injected into each muscle using a ¼ inch, 27 gauge needle. Fifteen minutes after the injection of the test material,

* The authors wish to thank M. L. Pabst for his advice and O. S. Carpenter for advice on statistical treatments.

0.3 cc of 1% Evans blue in saline was injected intravenously in the tail vein. Thirty minutes later the animal was sacrificed, both gastrocnemius muscles removed and placed in a semi-micro Monel blendor jar containing 15 cc benzalkonium chloride (Zephiran Chloride, 12.8%). The tissue and Zephiran Cl were blended at high speed in a 2-speed Waring blendor for 2 minutes, 13 cc isobutyl alcohol were added, and blending was continued for 30 seconds on low speed. The mixture was transferred to a small glass-stoppered reaction tube and centrifuged at 1750 r.p.m. for 3 minutes. After centrifugation, 3 layers were visible: a top layer of isobutyl alcohol containing the dye, a middle layer of white macerated tissue, and an aqueous-Zephiran Cl bottom layer.† Using vacuum, the 2 bottom layers were aspirated and discarded. Approximately 3.5 g sodium sulfate anhydrous were added to the remaining isobutyl layer to remove excess water and the tube was shaken by hand for one minute. It was centrifuged at 1750 r.p.m. for 5 minutes, and the supernatant decanted into a cuvette. Absorbency readings were made at 620 mμ in a Coleman Junior spectrophotometer. By means of a standard dye curve, the absorbency readings were converted into micrograms (μg) of dye.

Experimental. To ascertain the extent of dye recovery from the muscle tissue, the 3 layers of the first centrifugation were examined. Spectrophotometric analysis of the aqueous layer indicated that no dye was present. Microscopic examination of the macerated tissue layer showed no trace of the dye. Spectrophotometric analysis showed that the Evans blue was contained in the isobutyl layer. Therefore, this indicated quantitative recovery of the dye. The standard dye curve was prepared by processing increments of Evans blue, 10 to 150 μg in Zephiran Cl, according to the preceding method. The curve followed Beer's law confirming the work of Caster, *et al.*(13); therefore, it was used to convert the absorbency readings of all tissue extractions to μg of dye. The amount of dye and extraneous components extracted from non-irritated muscles after intravenous injection of Evans blue was determined as a "blank." The average blank for 22 rats was equivalent to 24.0 μg of dye. Because extraneous tissue components contributed to this value, the absorbency readings for normal muscles and test material injected muscles with dye absent were compared and found to be in agreement. Therefore, the blank was subtracted from all results obtained on the test materials to give a corrected value. Control injections of physiological saline and sterile water were tested and the results given in Table I. Physiological saline injections gave no evidence of irritation. Sterile water for injection was very slightly irritating which agrees with the findings of Weatherby(7).

TABLE I. Dye Measurements for the Blank and Control Injected Rats.

Materials tested	No. of rats	Dye value, avg μg/rat	Corrected dye value, avg μg/rat
Blank	22	24.0	—
Saline	16	23.7	−.3
Sterile water for inj.	6	27.9	3.9

Results. To test the procedure for graded responses to a known irritant, a series of concentrations of NF IX Formaldehyde were prepared and tested. The results are given in Table II. A regression line was calculated from the data in Table II and is shown in Fig. 1. Calculations of the regression line showed Slope = 27.1; Stand. Dev. = $\sqrt{107.16}$ =

TABLE II. Dye Measurements for Formaldehyde Concentrations.

Material tested	No. of rats	Corrected dye value, avg μg/rat
Formaldehyde 1.6 %	8	43.5
.8	8	39.3
.4	18	26.3
.2	10	18.7
.1	10	6.8
.05	10	7.1

† NOTE: Occasionally, contact of Zephiran Cl with the blendor assembly produced a blue color in the aqueous-Zephiran layer. A test for copper in this layer was positive. The blue color was insoluble in isobutyl alcohol, therefore, did not interfere with absorbency readings of the dye.

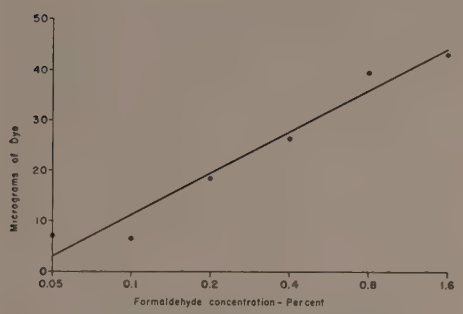

FIG. 1. Regression line for formaldehyde series.

10.352. The average dye measurements did not deviate from the regression line by more than could be attributed to experimental error. The slope of the regression line relating dye concentrations to dose concentrations was highly significant. Therefore, the method provides a quantitative measurement of degrees of irritation, assuming the degree of irritation to be proportional to the concentration of the irritant.

Preliminary examination was made of various categories of injectables to observe a range of irritation. The samples were tested at concentrations normally employed clinically. The results on materials examined to date are given in Table III.

TABLE III. Dye Measurements for Injectables.

Materials tested	Concentration	No. of rats	Corrected dye value, avg μg/rat
Preservatives			
Phenol	.5 %	3	–.9
Chlorobutanol	.5	3	1.6
Methylparaben	.2	3	2.2
Propylparaben	.02	3	1.9
Vehicles			
Cottonseed oil	—	4	4.1
Peanut oil	—	4	–.6
Al. Monostearate w/peanut oil gel	2.0 %	4	–.2
Local anesthetics			
Procaine HCl	2.0 %	8	3.4
Piperocaine HCl	2.0	7	1.4
Lidocaine HCl	2.0	8	4.3
Vitamin products			
Liver inj. (crude)	2 μg B_{12}/cc	8	11.1
Idem (refined)	10	11	5.3
" "	20	8	4.1
Nicotinic acid amide	10.0 %	4	5.6
Calcium compounds			
Ca. Gluconate	10.0 %	23	14.4
Ca. Chloride	2.5	13	35.9
Idem	1.25	10	24.0

The results in Table III indicate the preservatives, vehicles, and local anesthetics tested were only slightly irritating at clinical concentrations. The liver injection products did not cause irritation proportional to the amount of cyanocobalamin present; however, it appeared that refinement of the product reduced its irritating properties. Calcium chloride, which is not recommended for intramuscular injection due to its irritating properties, showed the highest degree of irritation in our study. Concentrations of 5.0 and 10.0% produced average dye values of 53.2 and 54.6 μg respectively. Because a significant increase was found in extracted extraneous components due to the irritant injection alone, these values were omitted from Table III. Dilutions of the calcium chloride from ¼ to ⅛ the clinical strength showed more irritation than calcium gluconate, which also had a high order of irritation.

Because of the current interest in anti-inflammatory compounds, a preliminary study was made to determine the adaptability of this method to measure anti-inflammatory activity. The procedure was modified only in that the test animals were injected with the anti-inflammatory material previous to the injection of the irritant, Calcium Gluconate, 10.0%. Intraperitoneal injections of Solu-Cortef‡ (hydrocortisone sodium succinate), 5 mg/rat, were used to obtain the anti-inflammatory effect. The preliminary results showed that a single dose injected 1 to 5 hours before the irritant would lower the amount of dye recovered by about 25%. This indicated that the method may be modified to measure quantitatively anti-inflammatory activity.

A preliminary test of xylene treatment of the rat abdominal skin indicated that a modification of the method may be used to measure quantitatively the irritation caused by agents applied topically.

Discussion. It appears that our preliminary results cover the range from non-irritating

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to highly irritating products. After the accumulation of more data, an attempt will be made to describe irritation on a unitage basis. At present, however, it is recommended that comparisons of irritation be made within categories of compounds, since there seems to be no universal agreement on a standard irritant. The same practice would probably apply to studies of the anti-inflammatory compounds.

In the course of this investigation, alternate sites of injection and modifications of the procedure have been used. The rat ear, rat testes, rat abdominal skin, and the longitudinal back muscle of the rabbit have been used successfully as an injection site for the irritant. In each case, the procedure of dye removal was modified slightly, but essentially the method remained the same.

Summary. An objective quantitative method to measure the degree of irritation has been described. It has been used to obtain a valid dose-response curve on a known irritant, formaldehyde, and to measure the irritating properties of various categories of injectables. The method has been modified to permit measurement of anti-inflammatory activity and topical irritation.

1. Ebbecke, U., *Klin. Wchnschr.*, 1923, v2, 1725.
2. Hirschfelder, A. D., *Am. J. Physiol.*, 1924, v70, 507.
3. Tainter, M. L., and Hanzlik, P. J., *J. Pharmacol. Exp. Therap.*, 1924, v24, 179.
4. Spagnol, G., *Arch. exp. Path. Pharmakol.*, 1928, v137, 250.
5. Menkin, V., *J. Exp. Med.*, 1929, v50, 171.
6. Tainter, M. L., Throndson, A. H., and Lehman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1937, v36, 584.
7. Weatherby, J. H., *J. Lab. Clin. Med.*, 1940, v25, 1199.
8. Rocha e Silva, M., and Dragstedt, C. A., *J. Pharmacol. Exp. Therap.*, 1941, v73, 405.
9. Last, M. R., and Loew, E. R., *ibid.*, 1947, v89, 81.
10. Hoppe, J. O., Alexander, E. B., and Miller, L. C., *J. Am. Pharm. Assn.*, 1950, v39, 147.
11. Caster, W. O., Simon, A. B., and Armstrong, W. D., *J. Lab. Clin. Med.*, 1953, v42, 493.
12. ———, *J. Applied Physiol.*, 1954, v6, 724.
13. ———, *Anal. Chem.*, 1954, v26, 713.

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Effect of Cyanide on Growth and Respiration in Earle's Strain L Cells.* (22100)

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Respiration in most tissues is inhibited by 3×10^{-3}M cyanide(3,8,12,14). Cyanide has been used very little as an inhibitor of cells grown *in vitro*. Danes and Leinfelder(2) noted that 0.1×10^{-4} to $.38 \times 10^{-4}$ M HCN inhibited growth of chick embryo tissue 73%. Experiments reported below were designed primarily to ascertain to what extent Earle's strain L cells, which have been grown in culture for more than 12 years(4), are cyanide sensitive.

Materials and methods. Strain L cells were obtained through the generosity of Dr. Wilton R. Earle and were cultured on glass (D-3.5 Carrel flasks) in Earle's solution consisting of 40% balanced saline, 40% horse serum and 20% chick embryo extract (in Earle's saline solution, 1:1)(4,5). The culture medium was renewed 3 times weekly and cultures gassed with 5% CO_2 in air. In experiments to test the effect of cyanide on growth, a cell suspension was obtained by gentle agitation of stock cultures. The cells were concentrated by centrifugation at 1000 rpm(7), resuspended in fresh medium after

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TABLE I. Effect of Sodium Cyanide on Growth in Earle's Strain L Cells. Growth of cells in control cultures is taken as 100.

Conc. of NaCN (molar)	No. of cultures	Avg No. of cell nuclei per culture	Growth (% of control)
1×10^{-3}	7	0	.0
1×10^{-4}	16	1.43×10^{5}	2.6
5×10^{-5}	9	2.19×10^{6}	99.6
1×10^{-5}	5	3.79×10^{5}	90.2
1×10^{-6}	20	1.88×10^{6}	95.5
1×10^{-8}	34	$1.98 \times$ "	117.3
1×10^{-10}	20	$1.96 \times$ "	116.2
1×10^{-12}	17	$2.21 \times$ "	94.6

which 1 ml aliquots were inoculated into Carrel flasks. After 2-3 days the cultures were divided into experimental and control groups. Sodium cyanide was added directly to the medium in experimental cultures which were carried for an additional week, at which time the number of nuclei in each culture was ascertained by sample counts on a hemacytometer(13). Cells were collected for respiration experiments in the same way as for growth experiments but they were resuspended in neutralized horse serum. Neutralized horse serum contains bound bicarbonate and remains physiologically neutral when equilibrated with a CO_2 free atmosphere(16) as is employed in Warburg's direct method (15). One ml aliquots were placed in each Warburg flask. Number of cell nuclei was determined in same manner as previously indicated. Calcium cyanide was prepared and its concentration determined according to Robbie(9,10). Constant source of HCN in medium during experimentation was obtained by using $Ca(CN)_2$-$Ca(OH)_2$ mixtures in center well of the Warburg flasks(11).

Results. Since number of cells used from one experiment to another was not exactly the same, determinations were made in pairs for both growth and respiration.

The results obtained on effect of NaCN on growth (Table I) indicate that Earle's strain L cells do not divide nor grow when placed in culture medium containing 10^{-3} M NaCN. After the first day in this solution all cells become detached from the floor of the flask. In 10^{-4} M NaCN the number of nuclei was scant or non-existent. Although some cells were still intact after a week it is doubtful that growth occurred in this concentration of cyanide. In culture media containing 10^{-8} M NaCN growth is stimulated. As checked by the test for observations involving paired groups(6), the accelerated growth in 10^{-8} M NaCN was significantly different from controls ($P =$ about 0.01). It appeared that growth might be stimulated in 10^{-10} M NaCN but statistically the results are not significant.

Results from experiments on effect of cyanide on respiration are presented in Table II. Oxygen uptake is inhibited 85.3% in 7 x 10^{-4} M HCN. It should be noted that a similar concentration of cyanide inhibited growth. Statistically, there was no evidence for stimulation of respiration with lower concentrations of HCN.

Discussion. Most of strain L cells did not grow in 10^{-4} M NaCN. In contrast, oxygen uptake was not completely inhibited in 2 x 10^{-4} M HCN; the mean oxygen uptake was 26.9% as much as the control. Probably difference in degree of inhibition of growth as compared to respiration can best be explained by the fact that the cells were exposed to cyanide for a longer period in the growth experiments.

It is known that the bulk of respiration is carried out by the cytochrome system. Cyanide inhibits cytochrome oxidase almost completely at concentrations of the order of 10^{-3} M, and thus inhibits about 80% of the respiration in most tissues(1).

Growth of embryonic chick heart in tissue culture is inhibited 73% in 0.1 x 10^{-4} to 0.38 x 10^{-4} M cyanide(2). The same is true for

TABLE II. Effect of Cyanide on Oxygen Consumption in Earle's Strain L Cells. Rate of oxygen consumption in control cultures is taken as 100.

Conc. of HCN (molar)	No. of determinations	Avg μl oxygen consumed/ hr/10^6 cells	Oxygen consumption (% of control)
7×10^{-4}	28	1.2	14.7
2×10^{-4}	28	2.0	26.9
6×10^{-5}	28	4.9	67.0
4×10^{-7}	24	7.7	109.2
2×10^{-8}	21	8.4	101.4
3×10^{-9}	28	7.5	100.4
2×10^{-10}	21	8.5	101.5
1×10^{-12}	24	8.1	105.8

other tissues. With slightly greater cyanide concentration (10^{-4} M NaCN), growth of strain L is inhibited 97.4%. Thus, although concentrations of cyanide in our experiments were slightly different from those used by other workers, the results are in agreement. Strain L is about as sensitive to cyanide as other types of tissue cells.

We noted that growth of strain L cells was stimulated with 10^{-8} M NaCN, as described by many investigators for other cells and tissues but no adequate explanation is available.

Summary. 1. Earle's strain L cells were cultured *in vitro* and the effects of different concentrations of NaCN were studied. Concentrations ranging from 10^{-3} to 10^{-12} M NaCN were tested. 2. In 10^{-4} M NaCN growth was inhibited 97.4% but in 10^{-8} M, growth was enhanced and exceeded that of controls by 17%. 3. The effect of cyanide on oxygen uptake of strain L cells in neutralized horse serum was determined by means of the Warburg Respirometer. 4. At a concentration of 7 x 10^{-4} M HCN, which was slightly stronger than the concentration necessary to stop growth, there was still an oxygen uptake of 14.7% as compared to controls. 5. Strain L cells possess a cyanide sensitive type of respiration similar to that found in normal cells.

1. Baldwin, E., Dynamic Aspects of Biochemistry. Cambridge, Cambridge Univ. Press, 1952.

2. Danes, B., and Leinfelder, P. J., *J. Cell. Comp. Physiol.*, 1951, v37, 427.

3. Dixon, M., Allen, K., and Elliott, C., *Biochem. J.*, 1929, v23, 812.

4. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

5. Earle, W. R., Evans, W. J., Sanford, K. K., Shannon, J. E., and Waltz, H. K., *J. Nat. Cancer Inst.*, 1951, v12, 563.

6. Edwards, A. L., Statistical Analysis. New York, Rinehart and Co., 1946, pp. 172-181.

7. Evans, V. J., Earle, W. R., Sanford, K. K., Shannon, J. E., and Waltz, H. K., *J. Nat. Cancer Inst.*, 1951, v11, 907.

8. Field, J., and Field, S. M., PROC. SOC. EXP. BIOL. AND MED., 1931, v28, 995.

9. Robbie, W. A., *Arch. Biochem.*, 1944, v5, 49.

10. ———, *J. Cell. Comp. Physiol.*, 1946, v27, 181.

11. Robbie, W. A., and Leinfelder, P. J., *J. Indust. Hyg. Toxicol.*, 1945, v27, 269.

12. ———, *Arch. Biochem.*, 1948, v16, 437.

13. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., *J. Nat. Cancer Inst.*, 1951, v11, 773.

14. Stare, F. J., and Elvehjem, C. A. Studies on the respiration of animal tissues. *Am. J. Physiol.*, 1933, v105, 655.

15. Umbreit, W. W., Burris, R. K., and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Minneapolis, Burgess Pub. Co., 1949.

16. Warren, C. O., *J. Biol. Chem.*, 1944, v156, 559.

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Eosinophil Response to Toxoids in Actively and Passively Immunized Mice.* (22101)

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The recent(1) development of technics for quantitatively estimating the number of cells in body cavity fluids has made it possible to determine the cellular reactions outside the blood following administration of hormones, drugs or other physiologically active substances. In a study of such reactions in the peritoneal exudate marked changes were found in the number of cells per cubic millimeter of peritoneal fluid depending upon a) the material injected, b) the degree of sensitization of the animal to that material, and c) the level of adrenal cortical hormones. An accumulation of eosinophils in the peritoneal fluid between the 2nd and 10th days appeared to be the specific local cellular response of the sensitized mouse to intraperitoneal injection of sensitizing antigen(1-3). The antigens used included pollen extract, bovine albumin, horse serum, ascaris extract and foreign erythrocytes.

This paper reports further studies concerning the local and systemic cellular responses to antigens and the chemotactic factors involved. It was found that injections of tetanus or diphtheria toxoid produced a local accumulation of eosinophils in actively immunized mice, but not in non-immunized mice or mice passively immunized with heterologous antibody.

Materials and methods. The mice used in these experiments were BBF_1 hybrids and C 57 Br/a mice from the research colonies of the R. B. Jackson Memorial Laboratory. The animals were from 2½ to 4 months of age and both sexes were used. The mice were immunized to diphtheria toxoid (Parke Davis and Co.) by a series of 5 subcutaneous injections as follows: 0.1 ml on days 1 and 2, and 0.2 ml on days 3, 6 and 13. The final challenging injection was given intraperitoneally one week after the last immunizing injection. Two different procedures were used for immunizing mice to tetanus toxoid. The first consisted of a series of injections of fluid toxoid (Lederle) as given above for diphtheria toxoid. The second consisted of injecting subcutaneously 0.05 ml of an alum phosphate precipitated toxoid (Lederle) followed by 0.05 ml fluid toxoid 15, 22 and 29 days later. On the 36th day a challenging injection of 0.05 ml fluid toxoid was given intraperitoneally. Passive immunization was produced by injecting mice with tetanus antitoxin (Lederle) either 1 or 72 hours prior to the injection of fluid tetanus toxoid. Two different dosages were used; half the animals were injected subcutaneously and the remainder intraperitoneally.

The methods used for estimating number of blood and peritoneal cells have been published elsewhere(1,4-6). A modification of Randolph's solution(7) was found to be a satisfactory diluent for the cell determinations. Each animal was used only once for the determination of cells and then discarded.

Results. The numbers of eosinophils in the peritoneal fluids of immunized and non-immunized mice, 4 days after intraperitoneal injections of toxoid, are compared in Fig. 1. In non-immunized animals a single injection of tetanus toxoid did not increase the number significantly. The immunized animals tended to have slightly higher numbers of eosinophils in the peritoneal fluid prior to the challenging injection. The animals which received the intraperitoneal injection of toxoid, however, showed as much as a 50-fold increase in these

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† Public Health Service Special Research Fellow of the National Cancer Institute.

IMMUNIZATION (SUBCUTANEOUS INJECTIONS)			FINAL INJECTION (INTRAPERITONEAL)		NO. OF MICE
MATERIAL	ML PER INJ	NO OF INJ	MATERIAL	DOSE (ML)	
NONE			NONE		9
NONE			TETANUS TOXOID	0.05	3
TETANUS TOXOID	0.05	4	NONE		6
TETANUS TOXOID	0.0008	5	TETANUS TOXOID	0.0008	5
	0.002	5		0.002	3
	0.02	5		0.02	3
	0.05	4		0.05	6
DIPHTHERIA TOXOID	0.002	5	DIPHTHERIA TOXOID	0.002	3
	0.02	5		0.02	3
	0.2	5		0.2	3

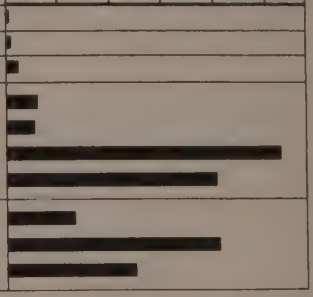

FIG. 1. No. of eosinophils in peritoneal fluid 4 days following intraperitoneal injection of toxoid in immunized and non-immunized mice.

cells. Similar data were obtained with diphtheria toxoid as the antigen.

The numbers of blood leucocytes and peritoneal cells observed following an injection of 0.05 ml of fluid tetanus toxoid in actively immunized and non-immunized mice is shown in Fig. 2. In the non-immunized mice, tetanus toxoid produced no marked changes in number of blood or peritoneal cells. However, in the actively immunized mice, there was a slight increase in blood eosinophils, and a marked increase in peritoneal eosinophils. A slight increase in the number of mononuclear cells and a transient neutrophilia also occurred.

The eosinophil responses in the peritoneal fluid on the 4th day following an intraperitoneal injection of 0.05 ml of tetanus toxoid in actively and passively immunized mice are compared in Table I. A marked eosinophilia

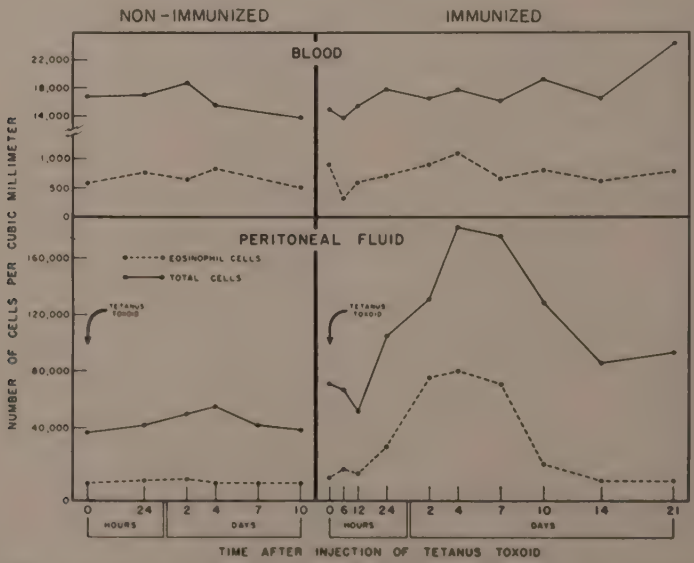

FIG. 2. Cellular responses following an intraperitoneal injection of tetanus toxoid in immunized and non-immunized mice. A total of 80 mice were used and each point represents the mean of 3 to 6 mice.

TABLE I. Comparison of Eosinophil Responses in Actively and Passively Immunized Mice 4 Days following an I.P. Injection of Tetanus Toxoid. 6 mice in each series.

	Procedure for immunization			Response to tetanus toxoid eosinophils/mm³ peritoneal fluid ± S.E.
Active	4 S. Q. inj. of 0.05 ml toxoid during 4 wk period			80660 ± 7817
Passive	A. 550 units antitoxin*	1 hr prior to toxoid†		1208 ± 166
	B. 2780	1	†	783 ± 271
	C. 550	72	†	1117 ± 127
	D. 2780	72	†	1158 ± 417

* Antitoxin—Lederle Globulin Tetanus Antitoxin (1 ml = 5560 units).
† Half of the mice injected IP, the remainder S. Q.

occurred in every animal which was actively immunized and then reinjected with the antigen. However, there was no evidence for an increase in the number of these cells in any of the mice passively immunized with antitoxin from a heterologous species.

Discussion. These experiments demonstrate that eosinophils accumulate in the peritoneal fluid following intraperitoneal injections of either tetanus or diphtheria toxoid into mice actively immunized to the respective toxoid. This local eosinophilia did not occur in control or passively immunized animals. The number of mononuclear cells also increased following the challenging injection of toxoid but the degree of increase was less.

It is well documented that antigenic material injected for the first time is rapidly removed from the injection site. However, in the actively immunized animal the antigen becomes fixed at the site of injection. This fixation is accompanied by antigen-antibody reactions, marked stimulation of antibody production, both locally, and in the draining lymph nodes, as well as an increase in the amount of local inflammation(8-10). It is shown in this paper that these reactions are also accompanied by an accumulation of eosinophils.

In the passively immunized animal also, there is fixation of antigen, antigen-antibody reactions, and local inflammation(9,10). However, an accumulation of eosinophils does not occur in these animals. It would therefore appear that the eosinophils are related to some phase of antibody production, since by the 4th day antibody production occurs in the actively immunized, but not in the passively immunized animal.

There has been a great deal of speculation as to which cells produce antibodies, but the eosinophil has not been considered by current workers or reviewers in this field despite earlier work suggesting this possibility(11-14). Eosinophils have been found to respond not only following the reinjection of toxoid, but also following the reinjection of a wide variety of particulate and soluble antigens, such as isoantigens, pollen extracts, albumins, horse serum, foreign red blood cells, parasitic extracts, etc.(1-3,15,16). The accumulation of these cells has been observed around the antigen whether it is introduced intraperitoneally, subcutaneously, intravenously, or via the digestive or respiratory tracts(3,17-20). In addition to the site of antigen injection, these cells also accumulate in lymph nodes draining an area of injection or infection and in the spleen following an intravenous injection of foreign protein(17,21). Furthermore, the time of local accumulation of eosinophils appears to precede slightly or to coincide with that of antibody production (22-24). It is also of interest to note that recent technics developed for staining antibody in plasma cells, also stain eosinophils in a similar manner(25,26). Finally, doses of cortisone and X-ray which inhibit antibody production also prevent the accumulation of eosinophils(27).

Summary. 1. Intraperitoneal injections of tetanus and diphtheria toxoid into actively immunized mice produce an accumulation of eosinophil cells in the peritoneal fluid which

reaches a peak at 4 days and lasts for at least 10 days. The degree of increase depends upon the dose of toxoid used. The local eosinophilia does not occur in non-immunized mice or in mice passively immunized with heterologous antibody. 2. The experiments suggest that the local eosinophilia is due neither to the antigen-antibody reaction nor to the accompanying inflammatory syndrome, but to an early stage of antibody formation during the secondary response.

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1. Speirs, R. S., and Dreisbach, M. E., *Blood,* 1956, v11, 44.
2. Speirs, R. S., Wenck, U., and Dreisbach, M. E., 1956, v11, 56.
3. Speirs, R. S., *Ann. N. Y. Acad. Sci.,* 1954, v59, 706.
4. Speirs, R. S., *Blood,* 1952, v7, 550.
5. Speirs, R. S., and Meyer, R. K., *Endocrinol.,* 1949, v45, 403.
6. Speirs, R. S., *J. Lab. Clin. Med.,* 1952, v39, 963.
7. Randolph, T. G., *Am. J. Clin. Pathol.,* 1944, v14, 48.
8. Menkin, V., *Physiol. Rev.,* 1938, v18, 366.
9. Kahn, R. L., *Ann. N. Y. Acad. Sci.,* 1955, v59, 281.
10. Opie, E. L., *J. Immunol.,* 1924, v9, 231, 255, 259.
11. Hankin, E. H., *Zentr. Bakteriol. Parasitenk.,* 1892, v12, 781.
12. Foster, G. B., *J. Med. Research,* 1908, v19, 83.
13. Schlecht, H., *Arch. exp. Pathol. Pharmakol.,* 1912, v67, 137.
14. Weinberg, M., and Sequin, P., *Ann. Inst. Pasteur,* 1915, v29, 323.
15. Panzenhagen, H., and Speirs, R., *Blood,* 1953, v8, 536.
16. Dreisbach, M., Snell, G., and Speirs, R., in preparation.
17. Schwarz, E., *Ergab. Allg. Path. u. path. Anat.,* 1914, v17, 137.
18. Essellier, A. F., and Koszewski, B., *Klin. Univ. Zurich,* 1951, v106, 10.
19. Samter, M., *Blood,* 1949, v4, 217.
20. Godlowski, Z. Z., *J. Endocrinol.,* 1952, v8, 102.
21. McNeil, C., *Am. J. Path.,* 1948, v24, 1271.
22. Libby, R. L., and Madison, C. R., *J. Immunol.,* 1947, v55, 15.
23. Koshland, M. E., *Fed. Proc.,* 1955, v14, 469.
24. Harris, S., and Harris, T. N., *J. Exp. Med.,* 1954, v100, 269.
25. Coons, A. H., Leduc, E. H., and Connolly, J. M., *ibid.,* 1955, v102, 49.
26. White, R. G., Coons, A. H. and Connolly, J. M., *ibid.,* 1955, v102, 73.
27. Wenck, U., and Speirs, R. S., papers in preparation.

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Thyroidal I^{131} Collection in Normal and Adrenalectomized Rats, Effect of Desoxycorticosterone Acetate and Physiological Saline.* (22102)

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Although it has often been suggested that changes in extrathyroidal distribution of iodine such as would be caused by body fluid shifts or variations in extracellular fluid volume will alter thyroidal I^{131} collection, little specific information on this point is available. Boatman *et al.*(1) reported an increase in thyroidal I^{131} concentration ratio 3 hours after injecting I^{131} in rats treated chronically with desoxycorticosterone acetate (DCA). It was postulated that this may have been due to the observed increase in blood radioactivity. In contrast to this, chronic treatment of rats with DCA was reported to cause no change(2) or a decrease(3) in 24 hour thyroidal I^{131} collection. In man(4) a decrease was also observed. Except for the report(5) that an increased amount of NaCl in the diet

* Data reported are from a thesis submitted by senior author in partial fulfillment of requirements for the degree of Doctor of Philosophy, Michigan State College, 1954, and published with approval of the Director, Mich. Agric. Exp. Station as Journal Article 1810.

TABLE I. Effects of DCA and 0.9% NaCl on 3 Hr Thyroid Uptake of I^{131} in Adrenalectomized Rats. 10 rats in each series.

Treatment	Body wt, g	Thyroid wt, mg	Thyroid I^{131}, % dose	Urine I^{131}, % dose	Plasma I^{131}, % dose/g	Muscle I^{131} "space,"† %
Controls	161	19.4	5.6	10.0	.75	19.3
DCA, 1.25 mg/100 g	162	17.4	8.8*	9.5	.69	19.4
0.9% NaCl, 5 cc/100 g	163	19.7	7.6	22.0	.52*	24.4*
" " , 10 "	164	18.3	9.6*	19.4	.45*	24.2*

* By analysis of variance significantly different from controls at 5% level.

† $\frac{\text{\% dose/g muscle}}{\text{\% dose/g plasma}} \times 89.2.$

of mice enhances the goitrogenic properties of the Remington iodine deficient diet, no reference to the effect of salt administration on thyroid function has been found. The present experiments were undertaken to determine the acute effect of DCA and physiological saline solution on short term thyroidal uptake of I^{131}. Concomitant measurements of blood plasma radioactivity and total urinary I^{131} output were made to determine the effect of the treatments on the extrathyroidal distribution of radioactive iodide.

Materials and methods. Eight to 10 days before the experiment, adult male albino rats received *ad libitum* an iodine deficient diet† with tap water to drink. The adrenals were removed at this time by a dorsal approach. The rats were given a daily subcutaneous dose of 0.25 mg DCA in corn oil. Completeness of operation was ascertained by gross examination for adrenal tissue at autopsy. Each rat received intravenously in a femoral vein exposed under light ether anesthesia 0.5 ml solution containing 20 uc I^{131} with 8.5 ug carrier I^{127} as NaI. Food and water were removed immediately before I^{131} injection. DCA, as a suspension in 0.1 to 0.2 ml corn oil and 0.9% NaCl solution (Merck Reagent), warmed to approximate body temperature, were injected intraperitoneally immediately after I^{131}. Exp. I contained 4 groups of adrenalectomized rats (Table I). The rats were placed by groups in metabolism cages for collection of urine; before removal of each rat from the metabolism cage the bladder urine was expressed manually. Rats were sacrificed by decapitation 3 hr after administration of I^{131}, and freely flowing blood collected into a beaker containing a drop of heparin. A muscle sample was obtained from the non-injected thigh. Exp. II contained 6 groups of rats (Table II). The animals were sacrificed 1½ hr after I^{131} injection, and blood was collected as before. Plasma and urine samples were weighed to the nearest mg in 1-in. pyrex dishes. A few drops of solution containing 1% NaI, 1% casein, 1% phenol and 1% $NaHSO_3$ were added to each sample to inhibit oxidation and sublimation of iodine. Thyroid glands were dissected cleanly, weighed rapidly to 0.1 mg and placed in the center of similar pyrex dishes. Aliquots from the injection solution were mixed with plasma for use as standards. After drying to constant weight, all samples were counted with an end-window Geiger-Muller tube and a laboratory scaler. Counts were corrected for physical decay, and in the case of plasma, urine and muscle, for self absorption of beta radiation. Results are expressed as % of injected dose.

Results. Rats receiving both DCA and the larger dose of physiological saline accumulated significantly increased amounts of I^{131} in their thyroids at 3 hr (Table I). DCA treated rats did not differ from controls in plasma radioactivity, % of dose found in their urine or in their muscle iodide space. The increased thyroidal I^{131} accumulation occurred in saline-treated rats despite the fact that their plasma radioactivity was significantly less and their muscle iodide space and urinary excretion of I^{131} was significantly greater than in either the control or DCA-

† "Iodine Deficient Diet," General Biochemicals, Chagrin Falls, O.

TABLE II. Effects of DCA and 0.9% NaCl on 1½ Hr Thyroid Uptake of I^{131} in Adrenalectomized and Intact Rats.

Group	Treatment	No. of rats	Body wt, g	Thyroid wt, mg	Thyroid I^{131}, % dose	Plasma I^{131}, % dose/g
Adrenalectomized	Controls	21	123	12.3	.66	1.20
	DCA, 1.5 mg/100 g	20	120	11.5	.65	1.16
	0.9% NaCl, 10 ml/100 g	20	120	12.1	.95*	.91*
Intact	Controls	21	133	12.9	.46	1.11
	DCA, 1.5 mg/100 g	20	129	11.7	.45	.99
	0.9% NaCl, 10 ml/100 g	19	131	12.1	.55	.85*

* By analysis of variance significantly different from controls at 5% level.

treated groups.

The only significant intergroup difference in thyroid uptake in the second experiment (Table II) is the increase in thyroidal I^{131} uptake in the saline-treated adrenalectomized group. Radioactivity of the blood plasma was reduced significantly in both the adrenalectomized and intact saline-treated rats.

Discussion. In view of the precautions taken in these experiments to standardize iodine intake, the increased thyroidal accumulation of I^{131} after both DCA and physiological saline solution should represent an increased amount of inorganic iodine fixed. The early uptake of radioiodine by the thyroid is known to be influenced by the thyroid to plasma I^{131} ratio, by the rate of conversion of inorganic to organic iodine and perhaps by the rate of blood circulation through the gland. There is no good evidence to suggest from these data which of the 3 may have been augmented in the treated groups. In contrast to earlier observations(1) regarding the effect of chronic DCA treatment on I^{131} concentration ratio, the increase in 3 hr uptake of I^{131} after administration of DCA was observed without any increase in blood radioactivity. A most interesting situation existed in the saline treated animals in that both the 1½ and 3 hr uptake of I^{131} increased despite a significant decrease in plasma I^{131} level.

Whether it is valid to compare the effect on the thyroid of DCA and physiological saline solution cannot be shown from the data available. Yet, speculation is attractive as to whether DCA might have a typical inorganic ion or water shifting effect at the level of the thyroid itself that results in a changed capacity to concentrate iodine and that the saline solution might have simulated the changes produced by DCA.

Summary. Adrenalectomized male rats, fed an iodine deficient diet and maintained on ¼ mg DCA daily, were injected intravenously with I^{131} containing 8.5 μg of iodide carrier. Thyroidal collection of I^{131} increased at 3 hr after 1.25 mg DCA/100 g, but not at 1½ hr after 1½ mg DCA/100 g, the DCA being given intraperitoneally immediately after I^{131} in both groups. There was no change from controls in plasma I^{131} level or urinary I^{131} excretion. Under similar conditions, adrenalectomized rats showed increased thyroidal I^{131} collection 3 hr and 1½ hr after intraperitoneal injection of 10 ml 0.9% NaCl/100 g. This occurred despite decreased plasma radioactivity and increased urinary I^{131} excretion. Intact rats studied under the same conditions showed no change from controls in thyroidal I^{131} collection, but had less radioactivity in their blood plasma.

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1. Boatman, J. B., Russ, C., Sunder, J. H., Konnerth, A., and Moses, C., *Endocrinology*, 1952, v50, 134.

2. Paschkis, K. E., Cantarow, A., Eberhard, T., and Boyle, D., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 116.

3. Money, W. L., Kraintz, L., Fager, J., Kirschner, L., and Rawson, R. W., *Endocrinology*, 1951, v48, 682.

4. Zingg, W., and Perry, W. F., *J. Clin. Endocrinol. Metab.*, 1953, v13, 712.

5. Axelrod, A. A., Leblond, C. P., and Isler, H., *Endocrinology*, 1955, v56, 387.

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Cortically-Evoked Pinna Movement in the Cat. (22103)

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Ferrier's(1) observation that stimulation in the cat ectosylvian region evoked ear movement, led him to postulate that this region constituted the auditory area. Bremer(2) considered that such topographic overlap of sensory and motor representations of various body parts has functional significance in indicating a mechanism subserving simple cortical reflexes. Although Ferrier's postulated auditory area has been amply confirmed by histologic and neurophysiologic studies, there are data indicating that acoustic stimulation also evokes cortical potentials in areas outside the ectosylvian gyrus. Mickle and Ades(3) and Bremer(4) observed such evoked potentials in a region roughly corresponding with somatic sensory II. We noted another region of activation more medially situated in the suprasylvian gyrus(5). Maps indicating cortical area for pinna movement reveal some discrepancies in localization (Fig. 1)(1,6,7, 8,9). Actual data are scanty; *e.g.*, Woolsey and Walzl(8) merely state that the suprasylvian area enclosed by broken lines "is the 'motor' area for the pinna" (their Fig. 10) and, similarly, Claes(9) gives no data supporting her "area for auricular movements." Ward and Clark(10) reported, as infrequent finding, that stimulation of anterior end of middle suprasylvian gyrus produced movement of the contralateral ear.

In studies of suprasylvian auditory response area our observations indicate a sensory-motor overlap, which reconcile, to some degree, discrepant findings in earlier reports.

Methods. Observations were made on 10 adult cats anesthetized with Dial-urethane, 50 mg/kg, or Nembutal, 35-50 mg/kg intraperitoneally, and on 2 modified "encéphale isolé" preparations. In the latter, operative procedures were carried out under ether anesthesia and the animal allowed to regain consciousness under artificial respiration after transection of the cord at C3 and infiltration of operative sites with procaine. The C3 level, rather than the bulbospinal level of Bremer, was chosen since a major portion of innervation of the pinna is derived from C1

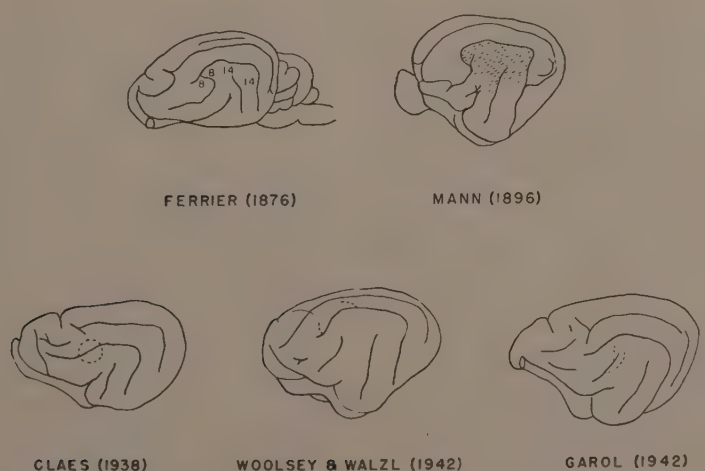

FIG. 1. "Motor area" for pinna movement as reported by other authors (reproductions of original diagrams).

and C2 segments. Various degrees of exposure of one or both hemispheres were made and stimulation carried out with bipolar platinum electrodes (inter-electrode distance 0.5-1 mm) leading from a Grass square wave stimulator. Optimal parameters of stimulation were determined and observations of pinna movements were recorded cinematographically. In some experiments in which the areas of acoustically-evoked potentials were defined, the electrocorticogram was recorded by a Grass Model III electroencephalograph, using wick electrodes.

Results. Exploration of lateral surface of cortex, made accessible by wide exposure, revealed that pinna movements were evoked from 2 small discrete areas, one in the ectosylvian gyrus (ES), lying within Auditory I area, and the other in the suprasylvian gyrus (SS) roughly corresponding with suprasylvian auditory receptive area mentioned above (Fig. 2). Since wide exposure of a hemisphere virtually destroys the ipselateral pinna, smaller exposures were made and care taken to preserve muscle and fascial attachments of the pinna as much as possible. It was then noted that the 2 responses differed significantly *i.e.*, ES response was always bilateral, whereas SS response was always unilateral, involving the contralateral pinna.

Actual movement of the ear varied somewhat from animal to animal, the most consistent pattern from SS stimulation consisting of an initial pricking up and cupping of the contralateral ear. If the stimulus was continued, this was followed by a "laying back" of the ear; *i.e.*, ear was retracted so that it lay in the plane of the dorsal convexity of the skull. With ES stimulation, the most common response was a pricking up of both ears, without cupping.

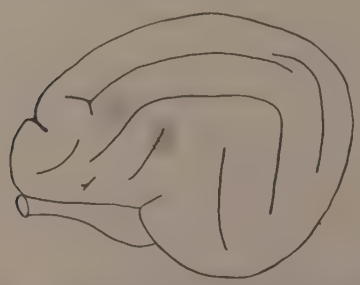

FIG. 2. Cortical areas from which pinna movements are evoked by electrical stimulation.

Both areas were approximately equal in size, and under conditions of these experiments covered approximately 20-30 sq mm in a given animal. No consistent differences in pattern of pinna movement could be determined by stimulation of different points within the responsive areas, even with closely spaced electrodes and threshold stimulus intensities.

The most effective parameters of stimulation for SS were 40-60/sec, pulse duration 5-10 msec, and 4-6 volts output. Pulse durations briefer than 2 msec rarely evoked responses. The required duration of stimulus was a few seconds. Changing parameters of frequency and pulse duration produced no significant changes in pattern of ear movement, if the stimuli were effective at all. With suprathreshold stimulation the required stimulus duration decreased, and initial pricking up of ears tended to disappear, movement consisting mostly of retraction. Antecedent stimulation frequently facilitated the responses from SS, so that with a series of stimulations, latency might be shortened to a small fraction of a second. In this way, a previously ineffective stimulus frequency of 5 or 10/sec might now become effective and evoke pinna twitches which followed the stimulus frequency. No marked extinction phenomena were noted.

ES required similar parameters of frequency and pulse duration, but in general had somewhat higher threshold than SS. In a number of experiments with good SS responses, no pinna movements could be elicited from any portion of the ectosylvian gyrus. The converse was not observed. When present, ES responses required somewhat higher voltages (6-10 v) and longer stimulus durations. No gross facilitation by antecedent stimulation was demonstrated for ES responses, which were also more susceptible to anesthesia, disappearing earlier than SS with increasing depth of narcosis.

Prolonged stimulation of SS frequently resulted in after-discharge manifested by

twitches of contralateral pinna. These seizures sometimes spread to involve contralateral face, but frequently remained limited to the pinna. Such post-stimulus effects were never observed after ES stimulation, despite use of strong and prolonged stimulation. Local application of strychnine to SS was followed by spontaneous twitches of contralateral ear, twitches being coincident with strychnine spikes as recorded electrocorticographically. Although less consistently obtained, strychninization of ES evoked bilateral ear twitches of lesser magnitude.

Ablation of ectosylvian or cruciate gyri did not influence responses obtained from SS stimulation, nor did ablation of SS abolish responses evoked by stimulation of ES.

SS responses were easily obtained from the modified encéphale isolé preparations, the fundamental pattern being similar to that observed in anesthetized animals.

Frequently, but not consistently, stimulation of SS evoked conjugate deviation of eyes to the opposite side, in addition to contralateral pinna movement. Such eye movements were not observed with ES stimulation, although reported to occur by Ferrier.

Discussion. There are 15 muscles which move the external ear of the cat; *i.e.*, change its orientation in space, and an additional 6 which change orientation of its parts to each other; *e.g.*, "cupping"(11). With such a complex of muscles, a considerable range of variability of movement pattern is conceivable. However, movements which have meaning to the human observer are limited to a few gross patterns such as pricking up of ears, as though "alerted," and laying back of ears, as though enraged. Behavioral interpretations of observed ear movements evoked by electrical stimulation face obvious difficulties. Nevertheless, the demonstration of unilateral responses from one cortical region as opposed to bilateral responses from another, represent two significantly different patterns of motor response.

It is difficult to determine, from reports of other investigators, whether any differentiation between bilateral and contralateral pinna movements has been made. It appears that other observers considered that ear movements represented an exploratory or alerting reaction to sound. Behaviorly, at least two types of auditory alerting must be considered. In one there is a generalized alerting; *i.e.*, both ears prick up and the animal becomes generally attentive without specific reference to the source of stimulus. This seems a response mediated by the ES area. In the other type, the animal becomes attentive to the particular region in space from which acoustic stimulus arises. One or both ears may respond, with the ear ipselateral to the stimulus source turning laterally or cupping, and head and eyes perhaps turning to that side.

Our studies of suprasylvian potentials evoked by acoustic stimulation indicate that these responses are largely evoked by stimulation of contralateral ear. Since this same area, when stimulated electrically, evokes pinna movement restricted to the opposite ear and, frequently, conjugate deviation of eyes to opposite side, it is suggested that this suprasylvian area mediates a cortical reflex concerned with "directed alerting"; *i.e.*, with an alerting reaction focussed toward the side from which acoustic stimulus originates.

Only part of the cortical efferent discharge evoked by afferent volley is directed towards motoneurones of brain stem or spinal cord, and a study of sensory-motor overlap gives information on only one aspect of function of such a region. Ability to localize sound in space has been attributed to asymmetrical activation of ectosylvian auditory area of the two hemispheres(12). The data here presented suggest that the suprasylvian region may also play a role in the mechanism of sound localization.

Summary. 1. Pinna movements in the cat are evoked by electrical stimulation of two discrete cortical areas. One, in the ectosylvian gyrus (ES) evokes movements of both ears; the other in the anterior suprasylvian area (SS) is of lower threshold, and evokes movement of the contralateral ear alone. 2. Both areas are topographically situated in regions in which acoustic stimulation evokes electrical responses. 3. The 2 areas of sen-

sory-motor overlap are considered to mediate 2 different cortical reflexes, the ES response of generalized alerting to unlocalized sound, and SS response one of oriented alerting to localized sound.

1. Ferrier, D. The Functions of the Brain. G. P. Putnam's Sons, New York, 1876.

2. Bremer, F. Some Problems in Neurophysiology. Athlone Press, London, 1953.

3. Mickle, W. A., and Ades, H. W., *Fed. Proc.*, 1951, v10, 92.

4. Bremer, F., *Rev. Neurologique*, 1952, v87, 65.

5. Merlis, J. K., and Lombroso, C. T., *EEG Clin. Neurophysiol.*, 1953, Suppl. #III, 70.

6. Mann, G., *J. Anat. and Physiol.*, 1896, v30, 6.

7. Garol, H. W., *J. Neuropath. and Exp. Neurol.*, 1942, v1, 139.

8. Woolsey, C. N., and Walzl, E. M., *Bull. J. Hopkins Hosp.*, 1942, v7, 315.

9. Claes, E., *C. R. Soc. Biol.*, 1938, v127, 1116.

10. Ward, J. W., and Clark, S. L., *J. Comp. Neurol.*, 1935, v63, 49.

11. Reighard, J. E., and Jennings, H. S. Anatomy of the Cat. Henry Holt & Co., New York, 1935.

12. Rosenzweig, M. R., *J. Comp. and Physiol. Psych.*, 1954, v47, 269.

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Mechanical Fragility of Human Neutrophilic Erythrophagocytes. (22104)

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It has been suggested that the erythrophagocyte may be a fragile cell(1) but controlled studies have not appeared in the literature. Studies presented here were designed to obtain quantitative data on this point as part of a study of properties of the erythrophagocyte and the nature of erythrophagocytosis. Erythrophagocytosis can be produced *in vitro*(2) by incubating phagocytic leucocytes with red cells sensitized by antibodies or other surface altering substances(3,4). A method for producing erythrophagocytes in high percentages in a uniform manner using iso-agglutinins was developed and used.

Definitions and method. For purposes of this experiment the erythrophagocyte is defined as any white blood cell which has ingested one or more red cells, red cell ghosts, or large fragments of either. Finely vacuolated cytoplasm in a leucocyte was not considered evidence for erythrophagia. White cells found erythrophagocytic under suitable conditions included neutrophiles, eosinophiles, and monocytes. This is in agreement with previous work(5,6). Of this group of cells only the neutrophile was present in appreciable percentages in the blood used, hence, our data applies to the neutrophilic erythrophagocyte. Cell survival time refers to *in vitro* period of morphologic integrity as indicated by presence of cell membrane or well defined cytoplasmic border seen by phase microscopy, chamber examination using special diluent, or on differential smear. Blood from normal, type A and O Rh negative white adult donors was used as source of red cells and anti-sera repectively. In plasma from type O donor, the anti-A titer was at least 1:512. The same red cell and anti-sera donors were used throughout. Anti-sera showed no agglutinating or lysing properties against the leucocytes used. White blood cells were obtained from normal type O adults and adult patients with elevated leucocyte counts and high neutrophile percentages. Blood was drawn in 5 cc samples to which 0.01 cc heparin (50 mg/cc) had been added. Erythrophagocytic activity does not appear to be altered by presence of anticoagulants (4,5). Blood from each donor was centrifuged 3 minutes at 2500 rpm promptly after drawing. The buffy coat from the leucocyte donor was removed with red cells and plasma to yield a mixture containing approximately 60% cells and 40% plasma by volume. Cells

TABLE I. Results of 14 Determinations in Normal Adults of 50% Survival Times (ST50) of Control and Experimental Neutrophiles Subject to Mechanical Trauma.

ST50 experimental	ST50 control	Ratio of ST50 control to ST50 exper.	% erythrophagocytes in total WBC	% neutrophiles erythrophagocytic	Total WBC
135	265	1.96	40	97	6850
144	289	2.01	48	100	6950
149	285	1.91	56	100	7400
135	255	1.89	60	99	6700
122	252	2.07	52	98	8050
108	250	2.31	66	96	7250
132	268	2.03	48	100	7250
144	295	2.05	58	100	8500
180	378	2.10	56	96	7650
180	360	2.00	68	99	6200
333	386	1.16	55	98	6950
144	351	2.44	64	100	7900
135	345	2.56	48	100	6950
117	304	2.60	56	96	6100

in this mixture were transferred to Wintrobe tubes placed in 37°C water bath, and tilted for rapid sedimentation by gravity to produce a leucocyte rich plasma. Final incubation mixtures placed in 5 cc test tubes consisted of 1.5 cc of anti-A plasma to which had been added 0.50 cc of leucocyte rich anti-A plasma and 0.025 cc of saline washed type A red cells. The leucocyte count in these mixtures varied from 8000-10000 cells/cu mm. When necessary, in samples from white cell donors with marked leucocytosis adjustment of cell count to this level was made by dilution with plasma from the same donor before adding the samples to incubation mixture. Mixtures were incubated 1 hour in 37°C water bath then transferred to 125 cc Erlenmeyer flasks, each containing six 3 mm round, glass beads, and placed on mechanical rotator at 180 rpm. The initial aliquot was taken prior to start of mechanical trauma. Temperature during period of trauma was maintained at approximately 35-37°C by placing flasks in a closed, insulated, electrically warmed box fastened to mechanical rotator. White cell counts and differential smears were obtained on initial sample and on each 0.15 cc interval aliquot. 300 consecutive white cells were counted for each differential. No attempt was made to differentiate between young and old neutrophilic forms of erythrophagocytes. Two separate pipettes and chambers were used for each cell count. All cells were counted using high power objective. A fresh solution of Giemsa stain in isotonic saline was employed as diluent. Turk's solution containing acetic acid or any hypotonic diluent tended to rupture erythrophagocytes. Our method left some red cells intact but their relatively small numbers did not interfere with leucocyte counts. Some clumping of white cells occurred after trauma began, as observed by others(7) but reproducible white counts were obtainable. Clumping was frequently marked if white cells other than those from type O donors were used. Comparison of differential counts made on smears with those made in a phase counting chamber using phase microscopy revealed close agreement showing that smearing did not measurably destroy erythrophagocytes. This was supported less directly by relatively slight decline in erythrophagocytic percentage in untraumatized control groups examined by smear. Control mixtures were employed with each series. The major control group contained anti-A plasma and white blood cells in the same quantity as in experimental group, however, type O red cells were substituted for type A to prevent their sensitization and subsequent ingestion by white cells. Other mixtures identical with those mentioned above but not subject to mechanical trauma were also used. In each instance, cells and plasma were obtained from the same donors as in experimental series.

Results. The results of 14 determinations using white blood cells from normal adult type O donors are shown in Table I. Total

white blood count of this group varied from 6100 to 8500 leucocytes/cu mm. In each case the number of stab and juvenile forms was less than 6%. Neutrophilic erythrophagocytes contained an average of over 2 ingested red cells, ghosts, or large fragments and at times as many as 9. About ¾ of these appeared to be ghosts. Following a Lepehne (8) stain for hemoglobin varying amounts of this pigment could be demonstrated in many red cell ghosts. Donor No. 1 provided antisera for the entire group as well as white cells for the first determination. Survival times of 50% of cells (ST50) were obtained from curves plotting percentage of remaining cells against time. In every instance ST50 of the control neutrophiles exceeded that of erythrophagocytic neutrophiles. To provide a measurement of relative fragility of cells under those experimental conditions the ratio of ST50 of the control to ST50 of experimental groups was tabulated (column 3, Table I). The average value of this ratio equaled 2.08 with standard deviation of 0.34.

In patients with leucocytosis and neutrophilia with varying degrees of "shift to the left" similar survival times were done. These are shown in Table II. The ratios for relative fragilities in this group were somewhat higher than for normals but of the same general order of magnitude.

The disappearance of experimental neutrophilic erythrophagocytes and control neutrophiles from 14 normal donors are shown in Fig. 1, based on average values obtained from determinations of absolute neutrophilic erythrophagocyte and neutrophile numbers derived by multiplying total white cell count of an aliquot with percentage of these cells in the differential smear of the same aliquot. Significant differences in disappearance of neutrophilic erythrophagocytes and neutrophiles were not found in those systems which did not receive mechanical trauma.

Discussion. Results indicate that within the artificial system employed, neutrophilic erythrophagocyte is about 2 times more fragile than neutrophile which has not ingested red cells or red cell ghosts. This means that antibody which is active against

TABLE II. 50% Survival Times (ST50) of Experimental and Control Neutrophiles Subject to Mechanical Trauma in 6 Patients with Leucocytosis and Neutrophilia.

ST50 experimental	ST50 control	Ratio of ST50 control to ST50 exper.	% erythrophagocytes in total WBC	% neutrophiles phagocytic	% juveniles and stabs in neutrophile group	Total WBC (× 1000)	Disease
117	380	3.26	92	99	42	24.5	Bronchiectasis
104	348	3.34	90	100	36	18.5	Cerebral hemorrhage & bronchopneumonia
108	290	2.69	90	98	50	16.5	? Pyelonephritis
126	370	2.94	86	97	36	21.0	Carcinoma of cervix with metastasis
147	384	2.62	88	100	64	28.0	Carcinoma of prostate and bronchopneumonia
117	450	3.84	88	98	32	14.5	Cirrhosis of liver

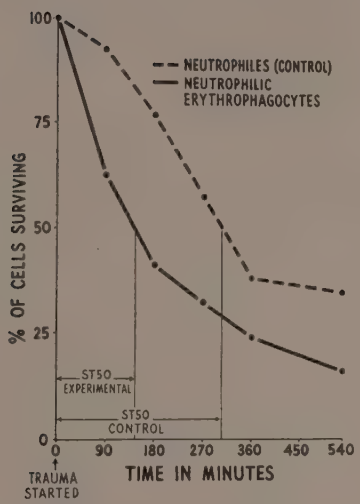

FIG. 1. Disappearance of neutrophilic erythrophagocytes and control neutrophiles subject to mechanical trauma with comparison of 50% survival times.

red blood cells can shorten survival time of neutrophiles present in the same system by promoting their conversion to fragile erythrophagocytic forms. This occurs in the absence of anti-leucocytic activity in the antibody. An exact measurement of this enhanced fragility, under these special circumstances, may be less important than the fact that fragility appreciably increases. Jordan (9) has summarized the theories relating frequent association of leucopenia and especially neutropenia with erythrophagocytosis *in vivo*. There are two main concepts: (1) entrapment of large erythrophagocyte in capillaries of the lung, liver, and spleen and (2) action of leucocytic lysins. It is suggested from our results that leucopenia from enhanced neutrophile fragility should be investigated as a possible explanation of this association.

Further, this enhanced fragility may help explain other findings associated with some acquired hemolytic anemias. It has been observed that frequently erythrophagocytosis is absent or minimal in films of peripheral blood in cases which have demonstrable red cell sensitization (positive direct anti-globulin test). However, if blood from the same sources is incubated *in vitro* at 37°C, large numbers of erythrophagocytes may be produced(1), suggesting that removal of circulatory trauma allows these mechanically fragile forms to survive, although other explanations can be given. While considerable emphasis previously has been on the role of phagocytes in reducing the number of circulating red blood cells, this study gives evidence for the importance also of the reverse consideration, namely, possible role of the red cell in reducing the number of circulating neutrophilic phagocytes.

Summary. Neutrophilic erythrophagocytes were produced in high percentages by sensitizing type A human red cells with anti-A isoagglutin in the presence of viable leucocytes. These erythrophagocytes were subject to mechanical trauma and found appreciably more fragile than neutrophiles which had not ingested red cells but which were treated in an identical manner. The possible significance of this decreased survival time is briefly discussed.

1. Zinkham, W. H., and Diamond, L. K., *Blood,* 1952, v7, 592.
2. Hektoen, L., *J. Infect. Dis.,* 1906, v3, 721.
3. Wright, C. S., Dodd, M. C., Brandt, N. G., Elliott, S. M., and Bass, J. A., *J. Lab. and Clin. Med.,* 1953, v41, 169.
4. Freund, J., PROC. SOC. EXP. BIOL. AND MED., 1929, v26, 876.
5. Bonnin, J., and Schwartz, L., *Blood,* 1954, v9, 773.
6. Conway, H., *J. Clin. Path.,* 1953, v6, 208.
7. Tullis, J. L. Blood Cells and Plasma Proteins, 1953. Academic Press, 265.
8. Sandoz Atlas of Haematology, 1952. Sandoz Ltd., Basle, Switzerland, 28.
9. Jordan, W. S., Prouty, R. L., Heinle, R. W., and Dingle, J. H., *Blood,* 1952, v7, 387.

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Dihydrostreptomycin Inhibition of Cysteine Desulfhydrase in Cell Free Extracts of *Escherichia coli*. (22105)

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The inhibitory effect of streptomycin on hydrogen sulfide production from organic and inorganic sulfur compounds by non-proliferating *Brucellae* and *Enterobacteriaceae* was reported by Olitzki(1,2). It was shown that preincubation of bacteria with streptomycin inactivates enzymes responsible for H_2S formation from various organic and inorganic sulfur compounds. These experiments were carried out using whole bacteria. The present paper examines the action of streptomycin on cysteine desulfhydrase in cell free extracts of *E. coli*.

Materials and methods. The organism used was *E. coli B/r strain*. Cysteine was determined by the method of Binkley and Nakamura(3). Streptomycin was assayed by the method of Sulitzeanu and Olitzki(4). This method is based on growth intensity of a streptomycin dependent strain of *Vibrio* and the inhibition by streptomycin of a sensitive one. Sulfide was determined by the method of Delwiche(5). Preliminary experiments having shown that cysteine desulfhydrase of *E. coli B/r* is an adaptive enzyme, the bacteria were grown on the surface of "Difco" nutrient agar in Roux bottles, to which cysteine at a final concentration of 0.1% was added aseptically. After 18 hours incubation at 37°, the cells were harvested in distilled water, centrifuged, twice washed in phosphate buffer (M/15, pH 7.2), disrupted by sonic vibration in a Raytheon Magnetostriction Oscillator for 15-20 minutes and centrifuged at 10000 rpm for 10 minutes. The supernatant solution was used in all experiments. Protein content of the extracts was determined by the biuret method in a Coleman Jr. spectrophotometer at 540 mμ, using a standard curve prepared for this purpose. Preliminary experiments on dihydrostreptomycin inhibition of H_2S production by cell free extracts of *E. coli* had shown a marked correlation between the degree of inhibition and the time of preincubation of streptomycin with the extracts before the addition of substrate. Preincubation of extracts with streptomycin for 15, 30, 60 and 90 minutes resulted in about 5, 13, 30 and 50% inhibition respectively. Preincubation of extracts with streptomycin for 2 and more hours caused almost complete inhibition of H_2S production. In all experiments reported in this paper the extracts were therefore preincubated at 37° for 2 hours with streptomycin and cysteine was then added. In order to ascertain that the loss of hydrogen sulfide production was not due to enzyme inactivation merely by incubation, in each experiment controls without streptomycin were subjected to the same incubation. All experiments were carried out in small test tubes closed by rubber stopper to prevent loss of hydrogen sulfide. For sulfide determination the reaction was stopped by the addition of sodium hydroxide. For cysteine determination the reaction was stopped by trichloracetic acid. In some experiments the extracts after preincubation with streptomycin were dialysed against distilled water. The dialysis was carried out in a cold room at 5°, with stirring for 18 and 35 hours before the substrate was added, the water being changed every 8 hours.

Results. Effect of streptomycin concentration on H_2S production from cysteine. To determine the minimal concentration of streptomycin required for almost complete inhibition of hydrogen sulfide production, 1 ml of extracts, equivalent to 7 mg protein, was preincubated for 2 hours with amounts of streptomycin ranging from 40 to 0.3 μg. After preincubation 10 μmoles cysteine was added. The pH of the reaction mixture was adjusted by phosphate buffer to 7.2 and sulfide was determined after 2 hours incubation at 37°. Samples without streptomycin were run as controls. The results (Table I)

TABLE I. Effect of Streptomycin Concentration on H_2S Production from Cysteine.

Amt of streptomycin (preincubated μg/7 mg protein)	Sulfide produced (μmoles)	% inhibition
40	.19	92
20	.19	92
10	.31	87
5	.70	71
2.5	1.4	42
1.25	1.58	35
.6	1.72	29
.3	1.82	25
0	2.43	0

Conditions as described in text.

show that the lowest concentration of streptomycin required for more than 90% inhibition of hydrogen sulfide production from cysteine was 20 μg per 7 mg protein.

Effect of cysteine concentration on H_2S production in presence and absence of streptomycin. Increasing amounts of cysteine were added to the extracts with and without preincubation with streptomycin in order to determine whether or not the inhibition of hydrogen sulfide production by streptomycin is of a competitive type. The results are summarized in Table II. Increasing the cysteine concentration until the system was saturated with the substrate caused a corresponding increase in hydrogen sulfide production by extracts not preincubated with streptomycin. The failure to produce hydrogen sulfide by preincubated extracts was not overcome by increase of cysteine concentration, indicating that the inhibition of hydrogen sulfide production from cysteine by streptomycin is not of the competitive type.

TABLE II. Effect of Cysteine Concentration on H_2S Production by Extracts of *E. coli* with and without Streptomycin.

Cysteine (μmoles)	Sulfide produced (μmoles) Without streptomycin	With streptomycin
10	4.10	.30
20	7.18	.30
40	8.12	.30
80	5.93	.26

Each vessel contained: Phosphate buffer (pH 7.2) 100 μmoles, cysteine as indicated, extract equivalent to 7 mg protein, streptomycin (when added) 20 μg. Sulfide was determined after 3 hr incubation at 37°.

To exclude the possibility that the loss of hydrogen sulfide production was due to some interaction between dihydrostreptomycin and cysteine the following experiments were carried out:

1. To extracts with and without streptomycin, cysteine was added and after 3 hours of incubation at 37° aliquots were tested for the presence of cysteine. The results showed that in the presence of streptomycin all added cysteine remained free and could be recovered. The following experiments with dialysed extracts served the same purpose.

2. Extracts without and in the presence of streptomycin (20 μg/7 mg protein) were dialysed as described above. After dialysis, cysteine was added and hydrogen sulfide determined after various periods of incubation. Dialysis for 35 hours caused about 40% loss of activity of cysteine desulfhydrase, in extracts without streptomycin. An almost complete loss of hydrogen sulfide production by preincubated extracts was observed when more than 75% of the original amount of streptomycin was removed by dialysis as shown in Table III. It was observed that after 18 hours of dialysis of an aqueous solution of streptomycin containing 125 μg in a volume of 5 ml, 5.5 μg/1 ml remained and after 35 hours of dialysis no streptomycin was left. When extracts con-

TABLE III. Effect of Dialysis of Extracts of *E. coli* on H_2S Production from Cysteine, with and without Preincubation with Streptomycin.

Previous treatment of extracts: Preincubation with streptomycin	Dialysis (hr)	Incubation with cysteine (hr)	Sulfide produced (μmoles)
Not preincubated		1	1.25
" "		2	2.12
Preincubated		1	.12
"		2	.15
Not preincubated	35	1	.63
" "	"	2	1.20
" "	"	3	1.79
Preincubated	"	1	.07
"	"	2	.10
"	"	3	.10

Each vessel contained: Extract equivalent to 7 mg protein, cysteine 10 μmoles, phosphate buffer (pH 7.2) 100 μmoles, streptomycin (when present) 20 μg. Final vol, 3 ml.

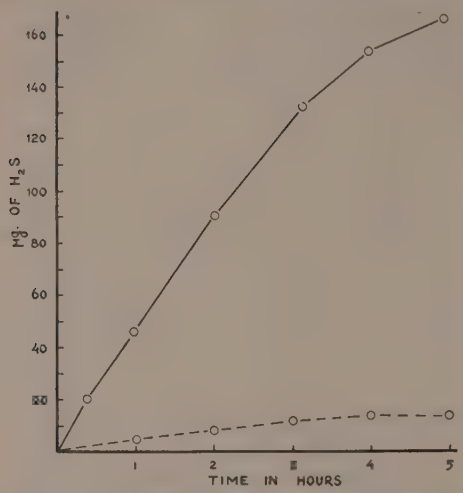

FIG. 1. The kinetics of H_2S production from cysteine by extracts of *E. coli* without and after preincubation with streptomycin. Each vessel contained: Extracts equivalent to 7 mg protein, cysteine 10 μmoles, phosphate buffer (pH 7.2) 100 μmoles. Final vol, 3 ml. Continuous line (———) H_2S production by extracts without streptomycin. Dotted line (- - - - -) *Idem*, with streptomycin (20 μg).

taining the same concentration of streptomycin (25 μg/1 ml extract equivalent to 10 mg protein) in the same total volume were dialysed under the same conditions, the amount of streptomycin left was 19.8 and 7.1 μg/1 ml respectively. These experiments showed that in the presence of extracts of *E. coli*, diffusion of streptomycin was retarded. However after 35 hours of dialysis the concentration of streptomycin was too low to give appreciable inhibition of hydrogen sulfide production from cysteine by non dialysed extracts. In spite of this there was almost complete inhibition of hydrogen sulfide production by dialysed extracts, indicating the irreversibility of cysteine desulfhydrase inhibition by streptomycin.

Discussion. It seems evident that low concentrations of streptomycin (20 μg/7 mg protein) caused almost complete and irreversible inactivation of cysteine desulfhydrase of *E. coli*. The inactivation became apparent after 2 hours preincubation of streptomycin with extracts. A shorter preincubation time caused a corresponding smaller effect. Similar observations were made by following authors: Geiger(6) observed that in *E. coli* streptomycin inhibited aspartate oxidation but only after 1-2 hours had elapsed. Umbreit and Tonhazy(7) had shown that streptomycin inhibition of oxygen uptake by rat kidney mitochondria in presence of fumarate and pyruvate was evident only when streptomycin has been in contact with mitochondria for a period of 2 hours in the cold before the substrates were added. Paine and Clark(8) had shown that the inhibitory effect of streptomycin on oxygen uptake by resting cells of *E. coli* became apparent after 1-2 hours incubation.

Summary. 1) The effect of streptomycin on the cysteine desulfhydrase in cell free extracts of *E. coli* is reported. 2) 2.5 μg of streptomycin/1 mg of crude extract protein caused almost complete inhibition of enzyme activity. 3) Inhibition is of a non-competitive type, since it is not reversed by increasing concentrations of substrate. 4) Inhibition is irreversible, since the removal of streptomycin by prolonged dialysis did not cause a restoration of enzyme activity.

1. Olitzki, A. L., *Atti del Congr. Internazionale di Microbiol., Roma,* 1953, v4, 186.

2. ———, *J. Gen. Microbiol.*, 1954, v11, 160.

3. Binkley, F., and Nakamura, K., *J. Biol. Chem.*, 1948, v173, 411.

4. Sulitzeanu, D., and Olitzki, A. L., *Exp. Med. and Surg.*, 1955, in press.

5. Delwiche, E., *J. Bact.*, 1951, v62, 717.

6. Geiger, W. B., *Arch. Biochem.*, 1947, v15, 227.

7. Umbreit, W. W., and Tonhazy, N. E., *J. Bact.*, 1949, v58, 769.

8. Paine, T. F., and Clark, L. S., *Science*, 1953, v118, 73.

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Rapid Freezing and Thawing of Whole Blood. (22106)

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Destruction of erythrocytes following freezing and thawing is so familiar a phenomenon as to have become a recognized procedure for obtaining complete red cell hemolysis. Only in very recent years have the basic principles underlying freezing in biology been sufficiently well established to suggest some of the causes of this hemolysis as well as means by which it might be avoided. It has long been recognized that there is a substantial difference between the results of rapid and slow freezing in biology. More recently this has been placed on a less empirical basis by the demonstration that slow freezing causes the formation of exclusively extracellular ice crystals which plasmolyze but do not necessarily rupture the cells while rapid freezing causes the formation of numerous intracellular ice crystals(1). When blood is slowly frozen, it has been shown that it is the dehydration and resultant electrolyte concentration which destroys the cell rather than the physical presence of the extracellular ice crystals(2). The success of glycerine in preventing the hemolysis of frozen erythrocytes can be attributed to the reduction in dehydration effected by the ability of glycerine to bind to itself and retain in solution much of the water which would otherwise have crystallized(3). Rapid freezing entails a considerably different problem and the only hope for subsequent survival lies in obtaining a crystal size sufficiently small to be benign despite the intracellular distribution. Luyet(4) has recovered morphologically intact erythrocytes after rapidly freezing and thawing a thin smear of blood on a cover slip but there are no other reports known to the authors in which the surface-to-volume limitations for rapid heat exchange were adequately recognized. Where the formation of extremely small intracellular crystals is tolerated it is presumed that the denaturing effect of removing water from solution can be reduced to an insignificant level by the rapid attainment of and storage at low temperature.

Since both the attainment of small ice crystals and the prevention of denaturation depend upon very rapid freezing to low temperature, the major problem is simply one of efficient heat exchange. Studies in the kinetics of freezing and thawing(5) have shown that the most efficient shape for heat exchange is the very small sphere and that the exchange of latent heat, particularly in thawing, is so demanding that every possible advantage that may be gained through specimen geometry must be exploited to the utmost if the transition from liquid to solid and back to liquid is to be sufficiently rapid. Further discussion of the theory and mechanism of the freezing of whole blood may be found in a more extensive paper on this subject to be published elsewhere(6).

Method. In view of the above remarks it can be seen that the first obstacle to successful rapid freezing of whole blood is the attainment of sufficiently fine droplet form without injury. Many means have been tried: the conventional atomizer, spinning disc and many types of jet, all with widely varying degrees of success. The method ultimately adopted was to spray the blood through a rapidly oscillating capillary jet drawn to an 0.18 mm aperture from 2.5 mm I.D. vinyl tubing. The fine droplets, ranging from .45 to .9 mm in diameter, are sprayed directly onto the surface of liquid nitrogen or liquid air where they float for a second, then sink to the bottom of the vessel. Reconstitution is achieved simply by sprinkling the frozen blood, which now resembles a fine sand, into plasma or physiological saline at 42°C. Throughout the developmental stages, spontaneous hemolysis was used as the criterion for erythrocyte injury.

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TABLE I. Post-Transfusion Survival of Erythrocytes after Freezing and Thawing Expressed as Percent of Whole Blood Radioactivity 15 Min. after Injection.

	Days post-transfusion																
Subject	¼	1	2	3	4	5	6	7	8	12	13	20	31	37	44	56	62
HM (autog.)	94	86	82	77	87		70	54			55	45	32	35	21	15	12
EK (homol.)	101	82	78	67	69	64			52	55		01					

Results. Following the standardization of the jet method as the least traumatic, recovery of intact erythrocytes was still rarely better than 50%. Because of these consistently poor although promising results, a compromise was introduced in the form of added dextrose designed to reduce the crystal growth rate(6) as well as to bind additional water and reduce the ultimate amount of ice formation. This produced an immediate improvement in percent recovery when added at the rate of 0.07 g/cc whole blood, reducing the spontaneous hemolysis to between 15 and 20%.

At this stage, 100 ml of dog blood were frozen, thawed, tagged with Cr^{51}(7) and reinfused into 2 dogs in order to determine the *in vivo* survival of the erythrocytes. Partly because of the fragility of dog erythrocytes and partly because of poor blood handling technic in this particular experiment, over 60% spontaneous hemolysis resulted from freezing and thawing alone. Of those cells remaining intact, only 18% were still present in the recipient circulation after 24 hours.

Following this equivocal result, every step of the procedure was reexamined. No major changes were made, but many minor improvements such as a change from polyethylene to vinyl tubing, covering the tubing with a rubber coat where it passed under the rollers of the peristaltic pump, making tubing and jet one piece instead of replaceable through a Luer-lok joint, and increasing the efficiency of heat exchange in the thawing bath, all contrived to reduce the damage to freshly drawn, citrated blood with added dextrose to consistently less than 3% spontaneous hemolysis after freezing and thawing. When dextrose was not added, 10 to 15% hemolysis could be expected. Inasmuch as another dog transfusion, whether positive or negative, would have to be confirmed in man, this intermediate step was bypassed and a reinfusion of Cr^{51} tagged blood was made in a human volunteer.

Two 100 ml aliquots of blood were drawn, each into a solution containing 0.32 g sodium citrate, 0.1 g citric acid and 7.3 g dextrose in 25 ml of solution. One of these contained, in addition, 1.0 μc of Cr^{51} as sodium chromate in 2 ml of solution. The tagged aliquot was frozen by the standard technic described above under experimental procedure. Fifty ml of plasma were removed from the untagged aliquot and used to cover the bottom of the reconstitution vessel. Because of the unaccustomed attempt to maintain a semblance of sterile technic, portions of the thawing procedure were clumsily handled and 6% hemolysis resulted. An aliquot of this blood containing an estimated 10 ml of cells was reinfused into the original donor by intravenous drip. Other than a brief psychosomatic response, no adverse clinical reaction was observed. Samples withdrawn 5, 10 and 15 minutes following infusion showed no change in radioactivity. A normal red cell volume calculated from these values indicated no immediate loss of cells after injection. At 6 hours a fall of 6% was observed. Subsequent samples taken daily for a week and at weekly intervals thereafter showed a slightly accelerated biological decay during the first 2 weeks with normal survival thereafter. (Table I). A second transfusion, this time homologous, is also charted in Table I. Mechanical failure again dogged the thawing apparatus and was presumably responsible for much of the 7% spontaneous hemolysis. Subsequent biological decay was again accelerated. Between the second and third week there was virtually a complete loss of blood radioactivity. A battery of tests failed to demonstrate any antigen in the donor's erythrocytes which might have been responsible. Preliminary experiments in rabbits indicate

that there may be an alteration in the attachment of chromium to the cell when tagging is done prior to freezing and thawing.

An *in vitro* examination of the blood platelets showed them to be substantially reduced in number as anticipated inasmuch as special precautions for platelet handling were not observed. Those remaining in whole blood after freezing and thawing appeared morphologically normal but were present in insufficient number for any test of function.

The following determinations were made on the clotting factors of the supernatant plasma: prothrombin time, recalcification time, accelerator globulin, antihemophilic factor, plasma thromboplastic component and serum prothrombin conversion accelerator. All were unaltered within the limits of experimental error compared to determinations on the same blood prior to freezing and thawing.

Storage. Only the most preliminary storage experiments have been undertaken. X-ray diffraction studies show complete crystallization in the frozen blood on the basis of which it is assumed that, while storage below about 130°C would probably be virtually indefinite, higher and more practical temperatures should still permit good survival for months or years, limited primarily by the rate of denaturation resulting from the dehydration of freezing. Exposures to temperatures as high as –20°C for a few minutes were found to be without ill effect. Preliminary results from longer storage at –60°C are difficult to interpret because of an accompanying acidification due to exposure to CO_2 from the dry ice coolant. Storage loss at –60°C was initially rather rapid, reaching 15% after 20 days (pH 6.5) but slowing subsequently to less than 2% per week. There is every expectation that the relationship between storage decay and temperature will be in accordance with the Arrhenius expression, showing a logarithmic decrease in decay rate with reduction in temperature.

Conclusions. Aside from premature speculation on the application of rapid freezing to blood preservation and storage, it can be unequivocally stated that, on the basis of *in vivo* survival, erythrocytes may be taken to liquid nitrogen temperature and back without destruction. The rather unusual fragility of the blood cell and the close relationship between its fragility and its state of health supports the impression that erythrocytes are particularly difficult to freeze. This in turn lends encouragement to the hope that other biological entities which can meet the rather stringent size requirements can be similarly frozen making available not only the benefits of long term storage, but providing also an intriguing new research tool through the study of transient phenomena interrupted and immobilized by the solid state.

Summary. 1. In accordance with previously derived criteria for non-traumatic rapid freezing, very small (.45-.9 mm) droplets of citrated whole blood with added dextrose are sprayed onto liquid nitrogen. The frozen blood is reconstituted in warm saline. 2. Two human transfusions of frozen and thawed whole blood containing erythrocytes tagged with Cr^{51} demonstrate the ability of red cells to survive *in vivo* after freezing in liquid nitrogen at –195.8°C.

1. Meryman, H. T., Naval Med. Research Inst., Project NM 000 018.01.08, 3 Jan., 1955.

2. Lovelock, J. E., *Biochim. et Biophys. Acta,* 1953, v10, 414.

3. ———, *ibid.*, 1953, v11, 28.

4. Luyet, B. J., *Biodynamica,* 1949, v6, 217.

5. Meryman, H. T., in preparation.

6. Meryman, H. T., and Kafig, E., Naval Med. Research Inst., Project NM 000 018.01.10, in preparation.

7. Ebaugh, F. G., Emerson, C. P., and Rose, J. F., *J. Clin. Invest.*, 1953, v32, 1260.

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Antibody Production in BALB/c Mice Following Injection of Lyophilized Tumor S621 in Freund's Adjuvant.*† (22107)

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Instances of isoimmunization of mice with their own tumors have recently been reviewed by Foley(1) and Snell(2). The criterion of immunization was ability of mice to cause regression of a viable implant of the tumor. Evidence was presented previously(3) for ability of strain BALB/c mice to produce antibody to homologous tumor S621. The criteria of antibody formation were ability of mice sensitized with lyophilized tumor to undergo anaphylactic shock on a second, intravenous injection; and ability of mice previously given tail inoculation of viable tumor, with tail and tumor subsequently removed, to cause regression of a second, subcutaneous injection of viable tumor.

The work reported herein was done to determine whether or not injection of lyophilized tumor in Freund's adjuvant provided a sufficient antigenic stimulus for detection of antibody by 3 technics: tumor regression; *in vitro* anaphylaxis by the Schultz-Dale reaction; and diminution of the number of exudate cells when cells were incubated with antigen and complement.

Experimental. Antibody production by protection against tumor growth. 1953 Experiments—Immunization with lyophilized S621: BALB/c mice from the colony of Dr. Flavia Richardson were used. Approximately equal numbers of both sexes, over 4 months of age, were injected intraabdominally as follows: (a) 20 mice injected with lyophilized S621‡ in Freund's adjuvant prepared as before(3) with the exception that only 50 mg of tumor were contained in the 0.5 ml injected; (b) 33 mice were injected with a preparation of lyophilized S621 identical with (a) except that the tumor in saline suspension was subjected to sonic treatment for 30 minutes in a Raytheon sonic oscillator immediately prior to mixing with adjuvant; and (c) 26 mice were injected with Freund's adjuvant equal in amount to that contained in the S621-adjuvant mixtures. *Injection of viable S621*: Five weeks following injection of lyophilized tumor, mice were inoculated subcutaneously with viable tumor prepared as follows: Fresh, healthy tumor tissue removed aseptically from host, and suspension of single cells prepared by Snell's sieve method(4). After making a cell count, the suspension was diluted with buffered glucose-Ringer's solution(4) to 150000 cells/ml. Each mouse was then injected subcutaneously with 0.1 ml, equivalent to 15000 cells. Twenty-two normal control mice were also injected. This dose was chosen because previous studies indicated that it exceeded, but not greatly, the minimum dose insuring tumor growth. Mice were examined at intervals for appearance of tumors. Experiment was terminated after 3½ months.

1955 Experiments—Mice were BALB/c from the colony of Dr. George Snell. Otherwise, they were comparable in every way to those used in 1953. They were injected with S621 under following conditions: (A) 17 mice were injected intra-abdominally with S621 in Freund's as described previously; (B) 16 mice were injected with the same preparation subcutaneously; (C) 11 mice were injected intraabdominally with 50 mg S621 suspended in 0.5 ml buffered saline; (D) 12 mice were injected with same preparation subcutaneously. Control mice included 11 injected with Freund's adjuvant intraabdominally; and 14 injected subcutaneously.

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† Part of this work was done while senior author was at Roscoe B. Jackson Memorial Laboratory (1953).

‡ A BALB/c fibrosarcoma induced by methylcholanthrene in 1948.

TABLE I. Effect of Prior Injection of Lyophilized S621 in Freund's Adjuvant on Growth of Viable Inoculum.

Material inj.	Date	Route of inj.	Progressively growing tumors; death	Slight, if any initial growth comp. regression	Total* by route	Total*† all routes
S621-Freund's	1953	IA	13/20*	7/20	18/37	32/53
	55	IA	5/17	12/17		
	"	SC	14/16	2/16	14/16	
S621-Freund's sonic	53	IA	28/33	5/33	28/33	28/33
S-621	55	IA	11/11		11/11	23/23
		SC	12/12		12/12	
Freund's	53	IA	25/26‡		36/37	50/51
	55	IA	11/11			
	"	SC	14/14		14/14	
None	53	—	22/22		40/40	40/40
	55	—	18/18			

* $\frac{\text{No. dead of tumors}}{\text{No. inoculated}}$

† No sex difference was ever apparent.

‡ One mouse showed moderate initial growth, followed by complete regression.

Five weeks after injection of lyophilized tissue, immunity of mice was challenged by subcutaneous injection of 30000 cells, prepared as before. Eighteen normal mice were injected as controls. Mice were examined as before, and experiment terminated after similar interval.

Results of the 2 experiments are recorded in Table I. They show that immunization of BALB/c mice with lyophilized tumor S621 in Freund's adjuvant provides sufficient antigenic stimulus to protect 21 of 53 mice from growth of a viable inoculum of tumor. When the immunizing injection was given intraabdominally, over 50% of the mice were immune. Injection of lyophilized S621 in saline did not protect mice against a subsequent injection of viable tumor. Sonic treatment of lyophilized S621 reduced antigenicity, but apparently did not destroy it.

Evidence of antibody production by in vitro anaphylaxis. Fifteen female BALB/c Jax mice were immunized with 50 mg of S621 in Freund's adjuvant as before. After 1 or 2 months, and 3 to 4 weeks before testing, they were given a small booster dose of S621 subcutaneously. Included in these experiments were 6 female survivors from the 1955 experiment previously reported. The technic for testing anaphylactic sensitivity in mouse uteri by the Schultz-Dale technic of *in vitro* anaphylaxis has been described(5). The test antigen was prepared from either lyophilized or fresh tumor. Lyophilized tumor was made to concentration of 50 mg/ml in special Ringer's solution(5), ground in glass, and the supernatant fluid, after centrifugation for 10 minutes at 3500 rpm, was used as antigen. Fresh tumor, which in general proved a little more potent antigen in this test, was prepared in like manner, with a crude attempt to approximate the concentration of lyophilized material. All materials were kept at 0°-4°C, and aseptic technic was used while preparing the antigen. After killing the mice and putting uterine strips in 45-ml water bath, 1.0 or 0.5 ml of antigen was added, and the reaction recorded on a kymograph. After contraction of the tissue, a second addition of antigen was made to test desensitization, and reactivity of the tissue tested by addition of 10 μg/ml of acetylcholine. Of the 15 BALB/c Jax mice tested, 8 gave positive reactions on at least two strips of uterus, and 7 gave negative reactions. Five of the 6 BALB/c Snell mice which survived the inoculation of viable cells

following immunization, gave positive reactions. Uterine strips from 3 normal BALB/c mice gave no reaction with either fresh or lyophilized antigen.

Evidence of antibody production by in vitro lysis of cells from sensitized mice. To become thoroughly familiar with the technic involved, preliminary work was done using egg white as antigen, as described elsewhere (6). Twenty-seven BALB/c Jax mice were immunized with 50 mg of lyophilized S621 in Freund's adjuvant, and tested 5 to 6 weeks later. Peritoneal exudate cells were obtained as follows: 2 ml of sterile mineral oil was injected intra-abdominally; 16-18 hours later, 2 ml of sterile heparinized serum-Fox solution(6) was injected. Ten minutes later cells were removed by aspiration, and put into chilled siliconed tubes. The cells were washed twice at 0°-4°C with serum-Fox solution, and resuspended in it to a concentration to give a count of 5000-20000 cells/mm^3. The S621 antigen used in the test was the supernatant fluid obtained after 50 mg/ml of lyophilized tumor had been ground in Fox solution and centrifuged 10 minutes at 3500 rpm. The control antigen was normal, lyophilized BALB/c liver, prepared in identical manner. Cells from 27 mice were tested individually. In each test, 6 siliconed tubes were set up, and the materials added to each in 0.1-ml amounts. Tubes 1 and 2 contained cells, S621, and complement;§ the controls contained serum-Fox solution instead of complement. The other 3 tubes were comparable except liver antigen was used instead of S621. After all additions had been made to each tube, which was shaken 100 times, a standard cell count made in National Bureau of Standards certified pipettes was counted in a certified counting chamber. The 6 tubes were then incubated for 2 hours at 37°C, after which time they were again shaken and another count made, using for each tube the same pipette. Data were collected for each of the 27 mice, and recorded as the percent difference in number of cells from 0 to 2 hours. For statistical analysis, results of duplicate tubes for each antigen, which showed good agreement in both cases, were averaged. Mean and standard deviation for each group were determined; and the statistical difference between experimental and control groups was determined by the *t* test. Results of this analysis are recorded in Table II.

§ The 1-10000 dilution of commercial lyophilized guinea pig serum was tested many times, and never had any deleterious effect on the cells.

TABLE II. Effect of Antigen and Complement on Peritoneal Exudate Cells from 27 S621 Sensitized BALB/c Jax Mice.

	Mean*	Stand. dev.	Difference†	t
Tests 1 and 2 (S621 antigen)	−11.4	7.71		
S621 control	3.0	7.63	14.4	6.73‡
Tests 3 and 4 (Liver antigen)	.9	7.76	12.3	5.75‡
Liver control	3.1	8.23	14.1	6.55‡

* Avg % difference in No. of cells at 0 time and 2 hr.
† Mean divergence from tests 1 and 2.
‡ Significant beyond .001 level of confidence.

From the results expressed in Table II, it can be seen that peritoneal exudate cells from mice sensitized by injection of tumor S621 in Freund's adjuvant were subject to lysis in the presence of antigen and complement; and that no such lysis occurred when normal tissue antigen from liver was used instead of the tumor.

Discussion. Work reported in a previous paper showed that strain BALB/c mice were capable of forming antibody to their homologous tumor S621. After injection of viable tumor in the tail, mice caused regression of a second implant of viable tumor. Some survivors from this experiment, as well as mice immunized by injection of lyophilized tumor in Freund's adjuvant, or lyophilized tumor alone followed by adrenalectomy, were anaphylactically sensitive to an antigen derived from S621.

Experiments here reported lend further support to the concept of formation of these antibodies. Mice immunized with lyophilized tumor tissue in Freund's adjuvant caused regression of subsequent viable implants; and uteri from female survivors of this experiment, as well as from other similarly immu-

nized mice, were anaphylactically sensitive as tested by the Schultz-Dale technic. The exudate cells of other mice, also sensitized with S621 in Freund's adjuvant, were lysed *in vitro* in the presence of the S621 antigen and complement.

With the exception of the technic of tumor regression, all our methods for detection of antibodies—*in vivo* anaphylaxis, *in vitro* anaphylaxis, and specific immune lysis of cells—are generally accepted immunological methods to detect "hypersensitive" antibodies, which in either immediate or delayed type of hypersensitivity reactions are believed to be in close association with cells. Recent work of Cole and Favour(7) has shown that in hypersensitivity of the guinea pig to tubercle bacilli, antibodies responsible for delayed "tuberculin" type hypersensitivity, as well as those of anaphylactic type, occur in the serum. Repeated attempts to find antibodies in circulation of BALB/c mice sensitized with S621 by various technics—precipitation, complement-fixation, hemagglutination of cells, which have or have not been treated with tannic acid prior to treatment with antigen, and passive protection with serum from immunized mice—have so far failed. Other studies of anaphylaxis in mice(8,9) indicate that a circulating, bivalent antibody is not the antibody concerned in anaphylaxis in the mouse. This leads to the tentative hypothesis that the antibody concerned in immunity to tumor S621 in BALB/c mice is not the conventional bivalent antibody, and even though it may be in circulation, is an antibody in close association with tissue cells.

Stewart(10) has presented a survey of spontaneous regression of various types of malignancies in man, many of which were accompanied by the classical signs of hypersensitivity in the host at time of regression of the tumor.

Immunity to S621 usually is accompanied by a state of hypersensitivity, but hypersensitivity can apparently exist in absence of sufficient immunity to protect the animal from viable cell growth. In a previous paper (3), as well as in present experiments, it is noteworthy that mice immunized with the lyophilized tissue in the absence of the adjuvant were not capable of causing regression of a viable implant of tumor, even though they had antibody demonstrable by the more sensitive anaphylactic technic. It is quite possible that lyophilized tissue alone did not provide sufficient antigenic stimulation for the formation of enough antibody to protect against relatively large challenge inocula.

Summary. 1. Antibodies were demonstrated in BALB/c mice which had received an injection of lyophilized homologous tumor S621 in Freund's adjuvant by the following technics: tumor regression; *in vitro* anaphylaxis using the Schultz-Dale reaction; decrease in the number of exudate cells when cells were incubated with antigen and complement. 2. Mice which recovered from a challenge injection of viable tumor were shown to react when tested by the Schultz-Dale technic.

We are grateful to C. D. Fink for the statistical analysis.

1. Foley, E. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 675.

2. Snell, G. D., in Physiopathology of Cancer, ed. F. Homburger, Paul B. Hoeber, Inc., N. Y., 1953.

3. Fink, M. A., Snell, G. D., and Kelton, D., *Cancer Research,* 1953, v13, 666.

4. Snell, G. D., *J. Nat. Cancer Inst.,* 1953, v13, 1511.

5. Fink, M. A., and Rothlauf, M. V., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 477.

6. Rothlauf, M. V., M.S. Thesis, 1954, University of Colorado.

7. Cole, L. R., and Favour, C. B., *J. Exp. Med.,* 1955, v101, 391.

8. Fink, M. A., and Rothlauf, M. V., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 336.

9. Olitsky, P. K., and Lee, J. M., *J. Lab. and Clin. Med.,* 1955, v45, 81.

10. Stewart, F. W., *Texas Rep. Biol. and Med.,* 1952, v10, 239.

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Inhibition Studies with *Streptococcus faecalis*. Xanthones and Thiaxanthones. (22108)

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Woods' finding(1), that the chemotherapeutic properties of sulfonamides resided in their ability to interfere with the utilization of p-aminobenzoic acid by the organism, has given tremendous impetus to the study of antimetabolites. Such studies, of course, may be approached from more than one direction. For example, anti-folics were prepared on the basis of their studied dissimilarity to the structure of folic acid. This gave rise to the useful agents aminopterin(2) and amethopterin(3). From another approach we have examined compounds prepared(4-6) for use against schistosomiasis(7) for their ability to interfere with utilization of folic acid by *Streptococcus faecalis*. The results of such tests on 16 xanthones and 63 thiaxanthones are discussed below.

Methods. The medium used for these studies with *S. faecalis* (ATCC No. 8043) was slightly modified from that of Teply and Elvehjem(8). The modifications consisted of omitting xanthine and of using "vitamin-free" casein hydrolysate (Nutritional Biochemicals) in place of charcoal-treated material suggested by the authors. Under these conditions the blanks required about 0.5 ml of N/10 alkali per tube for neutralization. Upon addition of 1 mμg of folic acid per ml of medium, growth of the organism, as judged by acid production after 72 hours, was represented by 9.5 to 10 ml of N/10 alkali per tube. Half maximum growth of the organism, the level against which the test compounds were run, resulted from addition of 0.32 mμg of folic acid per ml of medium. For the tests the compounds were added to duplicate tubes at levels of 1.6, 8.0, 40 or 200 μg per ml of medium. Tubes were plugged, sterilized at 120°C for about 12 minutes, cooled, inoculated with suspension of the organism(9), and incubated at 37°C for 3 days. Growth of the organism was estimated titrimetrically.

Results. The results summarized in Tables I-III are expressed as an arbitrary ratio term. The upper (and smaller) value represents μg of compound per ml of medium which had little or no inhibitory effect on growth of the organism. The lower (and larger) figure represents amount of test compound which completely or essentially completely inhibited growth of the organism. By way of reference, the ratio terms for some known anti-folics under these conditions of test were: (μg/ml) aminopterin 0.008/0.04, amethopterin 0.00032/0.0016, and pyrimeth-

TABLE I. Results of Tests with 16 Xanthones against *S. faecalis* in the Presence of Limiting Amounts of Folic Acid.

Compound,	8 1 7 CH=CH-CH-CO-CH=CH=CH 2 | ‖ ‖ | 6 CH=CH-CH-O—CH—CH=CH 3 5 4	Derivatives	Activity vs. folic acid, μg/ml
	1-NH$(CH_2)_2$N$(C_2H_5)_2$	4-CH_3 6-OCH_3, 4,6-$(CH_3)_2$	8/ 40
	N(C_2H_5) CH_2CHOH	4-Cl 6-CH_3, 4,6-$(CH_3)_2$,	"
		4-CH_3 6-Cl, 4-CH_3 6-OCH_3(4)	
		4-CH_3(4)	40/200
	NH$CH_2CHOHCH_3$	"	"
		4-CH_3 6-Cl(4)	8/ 40
	N(CH_3) CH_2CHOH CH_3	"	0
	NH CH_2CHOH C_2H_5	4-CH_3(4)	40/200
		6-Cl(4)	8/ 40
	NH$CH_2C(OH)$ $(CH_3)_2$	(4)	"
	N(C_2H_5) $CH_2CHOHCH_3$	6-Cl(4)	0
	N(C_4H_9) $CH_2CHOHCH_3$	"	20/100
	N$(C_2H_5)CH_2C(OH)$ $(CH_3)_2$	"	0

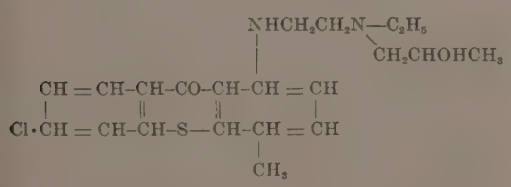
TABLE II. Thiaxanthones Which Inhibit *S. faecalis* in the Presence of Limiting Amounts of Folic Acid.

Compound, 8 1 / 7 CH=CH–CH–CO–CH–CH=CH 2 / 6 CH=CH–CH–S—CH–CH=CH 3 / 5 4	Derivatives	Activity vs. folic acid, μg/ml
1-NH$(CH_2)_2$NHCH$_2$CH$_2$OH	4-CH_3	8/ 40
NHCH$_2$CHOH CH_3	(5), 4-CH_3 6-Cl	"
NHCH$_2$CHOH CH_2OH	4-CH_3 (6)	"
NH CH_2CHOH C_2H_5	4-CH_3	"
"	6-Cl(5)	1.6/ 8
NH CH_2C(OH) $(CH_3)_2$	(5)	8/ 40
"	6-Cl	1.6/ 8
N(CH_3) CH_2CHOHCH$_3$	(5)	8/ 40
NC_5H_{10}	6-Cl	1.6/ 8
N$(CH_3)_2$	4-CH_3	8/ 40
N$(C_2H_5)_2$	(6), 4,6-$(CH_3)_2$	"
N(C_2H_5)CH$_2$CH$_2$OH	(5), 6-Cl(6), 4,6-$(CH_3)_2$, 4-CH_3 6-OCH_3, 4-$CH_3$6-Br	"
"	6-Cl(5)	1.6/ 40
N(C_2H_5) CH_2CHOH CH_3	"	1.6/ 8
N(C_4H_9) CH_2CH$_2$OH	(5)	8/ 40
N$(C_4H_9)_2$	4,7-$(CH_3)_2$(6)	40/200
N C_5H_{10}	4-CH_3	8/ 40
1-NH$(CH_2)_3$N$(C_6H_{13})_2$	(6)	"
1-NHCH(CH_3) CH_2N$(C_2H_5)_2$	"	"
1-NH$(CH_2)_3$N$(C_2H_5)_2$	"	"
"	4-CH_3 7-Cl (6)	1.6/ 8
1-NH$(CH_2)_3$N(C_2H_5) CH_2CH$_2$OH	(6)	8/ 40
1-NHCH$_2$CHOH CH_2N$(C_2H_5)_2$	4-CH_3 7-Cl (6)	"
"	2,4-$(CH_3)_2$(6)	40/200
1-NH$(CH_2)_3$ NH C_3H_7	4-CH_3(6)	8/ 40
N$(C_3H_7)_2$	"	"
N$(C_5H_{11})_2$	"	"
1-NH$(CH_2)_4$N$(C_2H_5)_2$	"	"

amine 0.0016/0.008.

From the data summarized in the Tables it may be seen that 13 of the 16 xanthones and 35 of the 63 thiaxanthones manifested some degree of toxicity to the organism. Since thymidine has been shown to abolish toxicity of some compounds which block utilization of folic acid(10), thymidine at 2 μg per ml of medium, was added in place of folic acid. The nucleoside level was otherwise adequate for optimum growth with the above medium.

In the thymidine-containing medium all xanthones and thiaxanthones inhibitory in the presence of folic acid were still inhibitory, with one exception. The exception was



Further tests showed that thymine(11,12) was unable to replace thymidine in abolishing the toxicity of this compound. This thiaxanthone thus differed from aminopterin, amethopterin, and pyrimethamine, which in the medium used, exhibited no adverse effects on growth of *S. faecalis* in the presence of thymine. The latter fact has been noted previously by others(13). On the other hand, the behavior of this thiaxanthone may be considered similar to that noted by Shive *et al.* (10) with another organism, who observed that thymidine, but not thymine, was able to abolish toxicity of either the sulfonamides or 2,4-diamino-6,7-diphenylpteridine for *L. arabinosus* 17-5. Whether or not the unusual metabolic blocking property of 6-chloro-1-{2-[ethyl - (2 - hydroxypropyl)amino]ethylamino}-4-methylthiaxanthone will prove useful alone or in combination with other blocking agents must await further studies under way.

In contradistinction to the unusual proper-

TABLE III. Thiaxanthones Which Do Not Interfere with the Utilization of Folic Acid by *S. faecalis*.

Compound,	Derivatives
8 1 7 CH=CH–CH–CO–CH–CH=CH 2 | ‖ ‖ | 6 CH=CH–CH–S—CH–CH=CH 3 5 4	
1-NH$(CH_2)_2$N(CH_3)CH$_2$CHOHCH$_3$	4-CH_3 6-Cl (5)
NC$_4$H$_8$O	4-CH_3 6-Cl (6)
N$(C_2H_5)_2$	4-NO_2 6-Cl (6)
N(C_2H_5)CH$_2$CH$_2$OH	4-CH_3 5-Cl (6)
N(C_2H_5)CH$_2$C(OH) $(CH_3)_2$	6-Cl (5)
N$(C_3H_7)_2$	(6)
N(C_4H_9)CH$_2$CHOHCH$_3$	4-CH_3 6-Cl (5)
N$(C_4H_9)_2$	4-NO_2, 4,6-Cl_2, 4,6-$(CH_3)_2$, 5-Cl(6), 4-CH_3 6-Cl, 4-CH_3 7-Cl(6), 7-NO_2(6), 4,5,7-$(CH_3)_3$, 4-Cl 5-CH_3(6), 4-Cl 5,7-$(CH_3)_2$, 4-Cl 7-NO_2, 4-NHCOCH$_3$(6), 4-NHCOC$_6$H$_5$(6), 4-NHSO$_2$C$_6$H$_5$(6)
N$(C_4H_9)_2$	4-CH_3 (6)
N$(C_5H_{11})_2$	"
N$(C_6H_{13})_2$	"
1-NH$(CH_2)_3$N$(C_4H_9)_2$	"

ties of thiaxanthone above, its xanthone analog given in Table I exhibited no adverse effects on the organism in the presence of folic acid.

It may be noted that the 4 thiaxanthones indicated by Berberian, Dennis, and Freele (7) to be schistosomacidal behaved quite differently from each other when examined for toxicity with *S. faecalis*. Thymidine was unable to reverse the toxicity of one (6-chloro-1-{2-[ethyl-(2-hydroxyethyl)amino]ethylamino}-4-methylthiaxanthone). Thymidine abolished the toxicity of one (6-chloro-1-{2-[ethyl(2-hydroxypropyl)amino]ethylamino}-4-methylthiaxanthone), as indicated above. The remaining two (1-(2-dibutylaminoethylamino)-4-methylthiaxanthone) and (6-chloro-1-[2-ethyl-2-(2-hydroxy-2-methylpropylamino)ethyl-amino]-4-methylthiaxanthone) exhibited no adverse effects on growth of the organism. Presumably, therefore, the schistosomacidal properties of the foregoing 4 thiaxanthones do not reside in their ability to interfere in the nucleic acid metabolism of *S. mansoni*.

We may conclude, accordingly, that one compound of interest, 6-chloro-1-{2-[ethyl(2-hydroxypropyl)amino]ethylamino}-4-methylthiaxanthone, has been brought to the fore by our study. While it does not compare favorably as an anti-folic with known anti-folics, being only 1/200, or less, as active as some of the better known ones, its differing pattern of inhibition points it up as a compound of possible interest and application, alone or in combination with other blocking agents.

Summary. 1. A series of xanthones and thiaxanthones initially prepared for tests against schistosomiasis was tested for toxicity with *S. faecalis* in the presence of limiting amounts of folic acid. Under these conditions 13 of the 16 xanthones and 35 of the 63 thiaxanthones tested were toxic. The toxicity of only one thiaxanthone could be abolished. 2. 6-Chloro-1-{2-[ethyl(2-hydroxyproyl)amino]ethylamino}-4-methylthiaxanthone was inert with thymidine in the medium. Thymine was not able to replace thymidine in this respect. 3. Separately, it may be indicated that no correlation was discerned between activity against *S. faecalis* and *S. mansoni* infection in mice.

1. Woods, D. D., *Brit. J. Exp. Path.*, 1940, v21, 74.

2. Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, v69, 2567.

3. Smith, J. M., Jr., Cosulich, D. B., Hultquist, M. E., and Seeger, D. R., *Tr. New York Acad. Sci.*, 1948, v10, 82.

4. Archer, S., Rochester, L. B., and Jackman, M., *J. Am. Chem. Soc.*, 1954, v76, 588.

5. Archer, S., U. S. Pat., 2,653,950 (1953).

6. Archer, S., and Suter, C. M., *J. Am. Chem. Soc.*,

1952, v74, 4296.

7. Berberian, D. A., Dennis, E. W., and Freele, H. W., *Communications, Congress. Internat. Med. Trop. Paludisine,* Istanbul, 1954, v2, 292.

8. Teply, L. S., and Elvehjem, C. A., *J. Biol. Chem.,* 1945, v157, 303.

9. Black, T. L., and Arnold, A., *Ind. Eng. Chem., Anal. Ed.,* 1940, v12, 344.

10. Shive, W., Eakin, R. W., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.,* 1948, v70, 2299.

11. Stokes, J. L., *J. Bact.,* 1944, v48, 201.

12. Hutchings, B. L., Mowatt, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.,* 1947, v170, 323.

13. Stokstad, E. L. R., Regan, M., Franklin, A. L., and Jukes, T. H., *Fed. Proc.,* 1948, v7, 193.

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Neutralizing Antibody to Viruses of Poliomyelitis in Sera of Domestic Animals. (22109)

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The explanation for the origin of seasonal outbreaks of poliomyelitis in the temperate zone is unknown, although it is now evident that man to man transmission is responsible for maintenance of an epidemic. Viral isolation studies have, as yet, failed to implicate animal-reservoir-insect-vector pattern which is so characteristic of many other neurotropic viruses. However, most isolation studies were done prior to the development of tissue culture technics, and intensive surveys were limited. Immunological studies have been carried out by Hammon(1) and others(2,3) in which "neutralizing factors" or "protective substances" have been found in sera of various animals. The status of these "protective substances" is uncertain for they were demonstrable only in low dilution and against one strain (Lansing, type 2), the only strain readily available in most laboratories. Whether one is here dealing with a non-specific neutralizing substance or specific antibody is a critical point, for identification of these substances as specific antibodies to viruses of poliomyelitis would point strongly to the existence of either (1) an extra-human reservoir of viruses of poliomyelitis or (2) a serologically related group of viruses. In the present study animal sera were tested for presence of neutralizing antibody to all 3 types of poliomyelitis virus using the HeLa cell. The protective substance existing in certain cases was identified as a true antibody. To determine the origin of this antibody, extensive though unsuccessful attempts were made to isolate the agents responsible for production of this antibody.

Methods. The HeLa Cell. Techniques employed in the management of the HeLa cell have been adequately described(4), and were followed in the present study. The HeLa cells were routinely grown in rubber stoppered test tubes in a growth medium consisting of 40% human adult serum, 2.5% chick embryo extract and 57.5% Hanks' balanced salt solution. Before use in neutralization test, the cells were washed 3 times with balanced salt solution and 0.9 ml maintenance solution was added to each test tube. The maintenance medium was composed of 90% maintenance solution as described by Syverton, Scherer and Ellwood(4) and 10% chicken serum. *Strains of Poliovirus.* The Brunhilde (type 1), YSK (type 2) and Leon (type 3) strains were employed as representatives of the 3 known antigenic types of poliovirus. These viruses were used in a 100 tissue culture dose concentration against each animal sera tested. *Source and Preparation of Serum Samples.* Table I illustrates the source of sera and geographical origin of animals from which sera were obtained. Sam-

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TABLE I. Number and Source of Animal Sera.

Animal	No. of sera	Source	Geographical origin
Cows	20	Slaughter house	Pa.
Steer	22	*Idem*	Midwest
Calves	18	"	Va.
Horses	28	Vet. School, Univ. Pa.	Pa. & N.Y.
Hogs	19	Slaughter house	Midwest
Chickens	20	Poultry market	Pa.
Lambs	19	Slaughter house	Va.
Dogs	26	Temple Med. School	Pa.
Cats	5	*Idem*	Unknown

ples of blood were collected in sterile tubes, allowed to clot, and refrigerated overnight. Serum was drawn off, inactivated at 56°C for one hour, and stored in a dry-ice box for approximately 6 months before testing. *Neutralization Test.* Serum dilutions were placed in sterile test tubes in 0.4 ml amounts to which was added 0.4 ml virus, of either types 1, 2 or 3, containing 100 tissue culture doses. The serum-virus mixtures remained at room temperature for one hour. Neutralization of the virus was determined by inoculating 0.1 ml of the mixture into 3 HeLa tubes. Tissue, animal serum toxicity and virus controls were routinely run. After 96 hours at 36°C the tubes were read for cytopathogenic effects. Highest dilution of serum completely inhibiting action of virus was the neutralizing titer of the serum. To determine if the neutralizing action was associated with the globulin fraction of the sera, globulins were precipitated out with a saturated solution of ammonium sulfate. The precipitated globulin was then diluted with distilled water to a final volume equal to that of original volume of sera. This was dialyzed and tested to determine if it had retained its protective activity against viruses of poliomyelitis. *Sources and Preparation of Tissue Specimens for Viral Isolation Studies.* In an attempt to establish the presence of a virus that might explain the origin of antibodies to viruses of poliomyelitis, samples of spinal cord, spleen, lymph node and feces were collected from cows, steers and calves. Specimens were collected as follows: approximately 1 cm^2 tissue, or 10 ml of feces were collected, 10 specimens from 10 animals pooled, and a 50% suspension prepared by grinding with mortar and pestle in tryptose broth containing 300 units/ml penicillin and 300 μg/ml dihydrostreptomycin. The suspension was then centrifuged at 3500-4000 rpm for 30 minutes, and the supernatant fluid tested for viral activity. Some tissue specimens proved to be toxic to HeLa cells, therefore the supernatant fluid of centrifuged suspensions was further diluted 1-5 in Hanks' balanced salt solution to reduce toxicity of test materials. One-tenth ml of each specimen was inoculated into 3 HeLa cultures in test tubes containing 0.9 ml maintenance solution. These tubes were incubated at 36°C and observed for cellular changes. After 5 days the fluid medium from each set of 3 cultures was pooled and 0.1 ml of this was inoculated into each of 3 HeLa cultures. This second passage also served further to dilute out toxic factors. After 4 days incubation at 36°C HeLa cultures were again observed for cytopathogenic changes. In addition, tissues were stained and observed for inclusion bodies to detect any agent which might have multiplied without producing a gross cytopathogenic effect. After removal of the media, HeLa cultures were stained directly in test tubes and on coverslips from Porter flask cultures with May-Grunwald-Giemsa stain.

Results. The data presented in Table II indicate type and number of animal sera having neutralizing activity against poliovirus. At a serum dilution of 1:2 some in-

TABLE II. Neutralizing Activity of Animal Sera against Polioviruses Types 1, 2 or 3.

Animal	No. of sera tested	No. of animals with neutralizing activity against polioviruses* Neutralization titer 1:2	1:10 or greater
Bovine			
Cow	20	5	14
Steer	22	3	6
Calf	18	5	0
Lamb	19	5	0
Chicken	20	6	2
Hog	19	3	6
Horse	28	6	9
Dog	30	5	0
Cat	5	0	0

* An animal serum was listed as a single positive if it neutralized types 1, 2 or 3 polioviruses or any combination of the 3 types.

TABLE III. Type Distribution of Neutralizing Activity against Poliomyelitis Viruses, Types 1, 2, and 3.

Animal	No. of sera tested	No. of positive reactions* Type 1	2	3	Total No. of positive reactions†
Bovine					
Cow	20	8	12	4	24
Steer	22	1	5	0	6
Calf	18	0	0	0	0
Horse	28	5	6	0	11
Hog	19	2	3	1	6
Chicken	20	1	1	0	2

* Only those sera exhibiting a neutralization titer of 1:10 or greater were included among the positive reactions.

† A single positive serum specimen may have contributed 1, 2 or 3 positive reactions depending on its ability to neutralize 1, 2 or 3 types of poliomyelitis virus.

dividuals of each species tested showed neutralizing activity, with exception of the cat. In many cases this neutralizing activity was lost upon further dilution. Since the neutralizing activity of undiluted or only slightly diluted serum may have been the expression of a non-specific inhibitory substance, only those species whose sera showed at least occasional activity at dilutions of 1:10 or greater were considered as possible poliomyelitis antibody producers. It will be seen in Table II that cows, steers, horses, hogs, and to a questionable extent, chickens possessed a capacity to neutralize one or more of the 3 types of poliomyelitis virus in serum dilutions of 1:10 or greater. On the other hand, sera of calves, lambs, dogs and cats were consistently negative at dilutions of 1:10 or higher, hence were not considered to be related to specific antibody. Type distribution of positive sera in the cow and steer is indicated in Table III. It will be seen that neutralizing activity against type 2 poliovirus was most frequently encountered followed by type 1, and least frequently against type 3.

TABLE IV. Titers of Antibodies in 20 Cow Sera against Poliomyelitis Viruses, Types 1, 2 and 3.

Cow sera	Titers of sera* Type 1	2	3
1	0	10	0
2	10	10	0
3	50	10	0
4	0	250	0
5	10	50	0
6	10	0	10
7	50	10	50
8	0	0	10
9	10	10	0
10	0	10	0
11	50	10	0
12	0	50	0
13	10	50	10
14	0	10	0
15-20	0	0	0
Total No. positive	8	12	4

* Expressed as reciprocal of actual dilution.

Probably the strongest evidence indicating that these neutralizing substances are antibodies is the pattern of neutralization observed with cow and steer sera as indicated in Tables IV and V. It can be seen that titers vary from 1:10 to 1:250 (the highest dilution tested) and that the pattern of neutralization tends to differ with each animal. Some animals have antibody to type 1, some to type 2, some to type 3, and still others have various combinations of these antibodies. This pattern, by definition, rules out the possibility of neutralizing action being due to a non-specific inhibitory substance. As further evidence that these are antibodies resulting from a specific antigenic stimulus and not a natural antibody it should be noted (Table III) that with increasing age there is an increasing ability to neutralize viruses of poliomyelitis. Thus, 70% of the cows (7-14 yrs), 27% of steers (1-2 yrs) and none of the calves (3-4½ months) had neutralizing antibodies in dilutions of 1:10 or higher. This increase with age is the typical pattern observed in antibody development. Table VI illustrates that this neutralizing activity is

TABLE V. Titers of Antibodies in Steer Sera against Poliomyelitis Viruses, Types 1, 2 and 3.

Steer sera	Titers of sera* Type 1	2	3
1	0	250	0
2	0	50	0
3	0	250	0
4	0	250	0
5	0	250	0
6	10	0	0
7-22	0	0	0
Total positive	1	5	0

* Expressed as reciprocal of actual dilution.

TABLE VI. Neutralizing Activity of Pooled, Positive Animal Sera and the Resuspended Globulin Fraction against Types 1, 2 and 3 Polioviruses.

Animal sera, pooled	Neutralizing activity: Before globulin precipitation, Type 1	Before globulin precipitation, Type 2	Before globulin precipitation, Type 3	Resuspended globulin fraction, Type 1	Resuspended globulin fraction, Type 2	Resuspended globulin fraction, Type 3
Cow	+	+	+	+	+	+
Steer	+	+	—	+	+	—
Horse	+	+	—	+	+	—
Hog	+	+	+	—	+	—
Chicken	+	+	—	—	—	—

(+) Neutralization. (—) No neutralization.

retained in the precipitated globulin fraction of sera.

Though extensive studies were done with bovine sera, preliminary data has been included on neutralizing activity of horse, hog and chicken sera (Table VII). It will be noted that both horse and hog sera were often able to neutralize in high dilutions, while chicken sera were able to neutralize only in low dilutions. These limited studies do suggest that sera of horses and hogs also possess specific neutralizing antibodies to viruses of poliomyelitis.

Viral Isolation Studies. One possible interpretation of the antibodies observed (see discussion) is the assumption that cows may be infected with all three types of poliovirus. If this assumption is valid, it should be possible to isolate the virus of poliomyelitis from the bovine species. Preliminary isolation studies have been carried out and no viruses were isolated in HeLa cells which were neutralized by types 1, 2 or 3 poliomyelitis antibody. These studies were carried out with materials collected from cows, steers and calves; 470 spinal cords, 330 spleens, 310 lymph nodes and 410 feces specimens were studied as indicated under Methods.

TABLE VII. Neutralizing Activity Exhibited by Horse, Hog, and Chicken Sera against Types 1, 2 and 3 Polioviruses.

Animal	No. of animals	No. of animals positive	Antipoliovirus titer and type distribution of positive sera*: Titer 1:10, Type 1	1:10, Type 2	1:10, Type 3	1:50, Type 1	1:50, Type 2	1:50, Type 3	1:250, Type 1	1:250, Type 2	1:250, Type 3
Horse	28	9	3	3	0	2	3	0	0	0	0
Hog	19	6	1	2	1	1	0	0	0	1	0
Chicken	20	2	1	1	0	0	0	0	0	0	0

* A single positive serum specimen may have contributed 1, 2 or 3 positive reactions depending on its ability to neutralize 1, 2 or 3 types of poliomyelitis virus.

Discussion. Our data indicate that neutralizing substances in bovine species are true antibodies for the reasons summarized below. 1. Antibody patterns in cows are highly specific, *e.g.*, some cows have no antibody, some have antibody to types 1 and 2, some to types 1 and 3, others to types 2 and 3, and still others have antibody to types 1, 2 and 3. This high degree of specificity, by definition rules out any non-specific neutralizing substance. 2. These are not natural antibodies for in addition to reasons stated above they are absent in young animals, increasing with age, and are not present in all adults of the species. In addition, titers are frequently high (1:250 dilution of serum neutralizes 100 TCD of virus). 3. Neutralizing substances are not inactivated by heating at 56°C for 30 minutes and are found in the globulin fraction of the serum.

It is therefore concluded that the neutralizing substances in cow sera are antibodies specific for polioviruses. It is important to note that these are antibodies *against* viruses of poliomyelitis and are not necessarily a result of infection with human strains of poliovirus.

While this study was in progress it was found that Dr. Albert Sabin (personal communication) was also carrying out a somewhat similar study(5). Dr. Sabin's data also indicate that neutralizing substances present in bovine sera are specific antibodies.

What is the origin of the antigenic stimulus? There are two possibilities: (1) These are antibodies resulting from subclinical infection of cows with human strains of poliovirus, with the implication that viruses of poliomyelitis exist and multiply in areas outside of man. (2). These antibodies arose not from the antigenic stimulus of human poliomyelitis strains but rather from viruses of non-human origin that are serologically related to human viruses. It is obvious that

such strains, if isolated, may be important as immunizing agents against poliovirus in humans. There is little experimental data to help us choose between these alternatives and little will be gained by further discussion of the possibilities since proof in either case requires the isolation from animals of the specific infectious agent.

Summary. (1) The sera of cows and steers were shown to possess neutralizing substances in high titer against the viruses of poliomyelitis. These have been shown to be specific antibodies against the viruses of poliomyelitis. (2) The sera of horses, hogs and chickens also possessed modest neutralizing activity against the viruses of poliomyelitis. (3) The sera of dogs, cats, calves and lambs do not possess neutralizing activity against the viruses of poliomyelitis. (4) Attempts to isolate a virus from the bovine species that might explain the origin of these antibodies were unsuccessful. The antigenic stimulus giving rise to antibodies against the viruses of poliomyelitis remains unknown.

1. Hammon, W. McD., Mack, W. N., and Reeves, W. C., *J. Immun.*, 1947, v57, 285.
2. Gordon, F. B., *J. Inf. Dis.*, 1945, v76, 198.
3. Gear, J. H. S., *Ann. Int. Med.*, 1952, v37, 1.
4. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.
5. Sabin, A. B., and Fieldsteel, H. A., 6th Internat. Cong. Microbiol., 1953, v2, 373.

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Myasthenia gravis. I. Importance of Potassium in Inhibitory Action of Normal and Myasthenic Thymus Extracts.* (22110)

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To investigate the concept that myasthenia gravis is due to a circulating blocking agent possibly elaborated by the thymus (McEachern)(1) several investigators(2-5) have tested thymus extracts on nerve-muscle preparations from various animals.

The present study was undertaken to repeat Dr. Wilson's experiments with the eventual objective of isolating the active blocking substance. The isolated frog sartorius nerve-muscle preparation, which had been used successfully by Wilson and Stoner(6) to detect the blocking activity of serum from myasthenic patients, was used in this investigation rather than the rat phrenic nerve-diaphragm preparation.

Methods. A. Isolated Frog Sartorius Preparation: Frogs were pithed and the sartorius muscle excised with a small block of underlying muscle to support the motor nerve. Frog Ringer, containing 6.5 g NaCl, 0.14 g KCl, 0.12 g $CaCl_2$, 0.2 g $NaHCO_3$ and 0.01 g NaH_2PO_4/l, bathed the muscle during dissection. After excision, the muscle was placed at normal resting length (5 to 10 g tension) in a plastic chamber of 2 ml capacity, provided with two sets of silver electrodes to permit independent direct and indirect stimulation of the muscle and nerve respectively. The small size of this chamber is of considerable advantage when only small volumes of test solution are available. The mechanical myogram was obtained by a transducer, consisting of an aluminum bar (6.5 x 1.2 x 0.015 cm) on either side of which was cemented a strain gage element.† The myogram was recorded with a Sanborn strain gage recorder (Model 127) fed from a Sanborn strain gage amplifier (Model 140). This arrangement permitted recording of the isometric myogram. All experiments were performed at 23 to 25°C. Thresholds for nerve and muscle excitability were recorded at beginning of each experiment. After 15 minutes equilibra-

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† Baldwin-Lima-Hamilton Corp., Type A7.

tion of the muscle in oxygenated frog Ringer, the Ringer solution was removed and the muscle stimulated via the motor nerve for 5 seconds with 0.5 to 1 volt monophasic stimuli of 0.5 millisecond duration at a frequency of 2 cps. A second and third control series of contractions were obtained at 5 minute intervals before adding test solution to the muscle chamber. At one to 3 minute intervals, the test solution was removed from the chamber and the muscle stimulated as before. When the muscle failed to respond to indirect stimulation, it was stimulated directly with 5 second trains of stimuli of increasing voltage (1,3, 5,10,14 volts). The muscle was then repeatedly washed with frog Ringer and recovery of muscle tension was followed for 60 minutes. Average amplitude of 10 consecutive tracings was computed and plotted against time. Activity of each extract was calculated on the basis of time for 50% inhibition of contraction strength per g of extracted thymus tissue.

B. *Method of extraction* was slightly modified after the method of Wilson, Obrist and Wilson(5). Thymus glands, removed at operation from patients with myasthenia gravis, were weighed and placed in cold acetone (4°C) after representative blocks had been taken for preparation of sections for microscopic examination. Each gland was chopped into small pieces and ground in a mortar with 25 ml volumes of cold acetone (4°C) until total extract volume of 1000 ml was obtained. Acetone extract (AE) was filtered and the residual insoluble material (AI) collected and dried to constant weight *in vacuo* over $CaCl_2$. The acetone fraction was evaporated to dryness *in vacuo* in a water bath at 37°C. After evaporation, the AE fraction which consisted of a yellow oil, was taken up in 5 ml of Frog Ringer. The red-brown, AI fraction was similarly taken up in 5 ml of frog Ringer. After adjusting the pH of the 2 extracts to the pH of frog Ringer, they were separately tested on the isolated frog sartorius preparation. A fresh sartorius muscle was used in each test. Sixteen thymuses from myasthenic patients, one normal child's thymus, 3 spleens and 2 samples of skeletal muscle were extracted and tested by this procedure. *C. Heat Inactivation and Dialysis Experiments*: Solutions of acetone insoluble fraction were placed in cellophane bags and dialyzed against frog Ringer. Aliquots of internal and external solutions were subsequently tested. Extracts of both acetone soluble and insoluble fractions were boiled for 2 minutes, cooled and tested on the sartorius preparation.

D. Experiments with d-Tubocurarine, $CaCl_2$ and KCl: d-Tubocurarine (0.003 to 15 γ /ml, and KCl (4 to 40 meq/L.) solutions were tested on the sartorius preparation. The effect of thymus extracts containing added $CaCl_2$ was also determined. *E.* The potassium concentration of 16 extracts was quantitatively determined by flame photometry.‡ Extracts of the acetone soluble fraction were ashed before determination of their potassium content because of fat in the solutions. *F. Thymus Pathology*: The presence of hyperplasia and germinal center formation was recorded (Castleman and Norris)(7) and the relative amounts of parenchyma, fatty and fibrous tissue was independently rated by two observers who were unaware of the activity of the extracts prepared from the glands.

Observations: *Control studies* performed

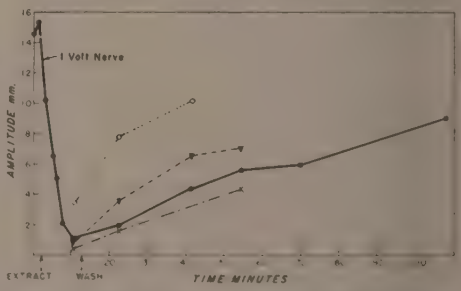

FIG. 1. Heavy circles illustrate decrease in contraction force of indirectly stimulated (1 volt) muscle resulting from immersion in thymus extract. After repeated washing, the contraction strength gradually returns. Crosses (1 volt), triangles (5 volts) and open circles (10 volts) illustrate that the muscle shows impaired response to direct stimulation. After washing, the curve of recovery of the directly stimulated muscle parallels the recovery curve of the indirectly stimulated muscle.

‡ Acknowledgment is due to Dr. Charles H. DuToit for these determinations.

TABLE I. Clinical Status of 16 Myasthenic Patients and the Activity of Extracts of Their Thymus Glands.

Age	Severity	Duration of symptoms	Activity of acetone insoluble fraction, min./g /50% inhibition
21	Mild	2.5 yr	1.33
25	"	1.5	.51
13	Moderate	3.5	.91
23	"	3.0	2.60
21	"	2.0	.20
15	"	1.5	1.33
16	"	1.0	.67
17	"	3 mo	1.14
19	"	5 yr	.73
30	Severe	7	.66
23	"	1	1.53
41	"	6	2.74 T*
13	"	2	.29
46	"	Months ?	.38 T
24	"	4.5 yr	.67
18	"	2	2.05

* T = Tumor.

with several changes of frog Ringer over periods of 60 to 70 minutes showed a maximal decrease in the tension curve of 15 to 20% of initial tension. When tissue extracts were tested, the average experiment rarely exceeded 30 minutes. Thus, decreases of the tension curve of greater magnitude are attributed to the inhibitory effects of the extracts. A dose-response curve constructed for several concentrations of d-Tubocurarine (0.003 to 15 γ/ml) showed that concentrations of 0.03 γ/ml of d-Tubocurarine could be accurately detected by this method. As expected, the muscle responded to direct stimulation; it failed to respond to indirect stimulation.

Experiments with Tissue Extracts: Both acetone soluble and acetone insoluble fractions of all thymus glands tested depressed the tension curve of indirectly stimulated sartorii in 2 to 20 minutes. However, when muscles failed to respond to indirect stimulation they also failed to respond to direct stimulation (Fig. 1). After 30 to 60 minutes of washing with several changes of frog Ringer, the test muscles slowly recovered. The time course of recovery for indirect and direct stimulation was similar (Fig. 1). Thus, it appeared that the extracts acted directly on the muscle, although action at the neuromuscular junction could not be excluded. Great variability of activity of extracts occurred which failed to correlate with age of patient, duration of symptoms or clinical severity of the disease. (Table I). A normal child's thymus possessed considerable inhibitory activity as did extracts of 3 spleens and 2 samples of human skeletal muscle. Extracts of these tissues rendered the test muscle inexcitable by indirect stimulation in 5 to 10 minutes. At this time, the muscles uniformly failed to respond to direct stimulation. The time course of recovery of the muscle after washing with several changes of frog Ringer was similar to that observed with thymus extracts.

Dialysis of Extracts: After dialysis of the acetone insoluble fraction, most of the inhibitory activity was present in the solution outside the cellophane membrane. This material produced a decrease of tension curves of both indirectly and directly stimulated muscles.

Heated Extracts: The acetone insoluble extract retained its inhibitory activity after boiling for 2 minutes.

Potassium concentrations of thymus extracts ranged from 2 to 33 times the concentration of potassium in frog Ringer. When the extract containing highest concentration of potassium (66 mEq/L.) was added to the test muscle, spontaneous fasciculations of the muscle occurred. These concentrations of potassium are 33 times the normal potassium content of frog Ringer. Table II shows a rough correlation between time for 50% inhibition of the muscle by the acetone-insoluble fraction and a poor correlation with the acetone soluble fraction.

Effect of Added Potassium Chloride. When

TABLE II. Potassium Content and Activity of Thymus Extracts.

Per g P_{50}	(K^+) P	TP_{50}	TA_{50}	(K^+) A
.727	2.0	8.0	—	—
.254	4.8	6.5	5.2	7.2
1.140	8.0	11.2	12.0	—
.671	11.0	8.0	3.0	7.7
.381	13.0	5.0	6.0	7.11
2.05	13.6	8.5	10.5	20.0
.287	17.0	8.8	1.9	6.1
2.740	38.5	3.7	4.7	24.3
1.330	42.0	2.9	—	—
.675	66.0	2.6	8.7	34.0

TABLE III. Potassium Content of Ten Thymuses Compared with Histological Architecture.

K content acetone soluble fraction, mEq/L	K content acetone insoluble fraction, mEq/L	Parenchyma	Fibrosis	Fat	Germinal center formation
2.0	—	1+	3+	2+	A*
4.8	—	3+	<1+	2+	P
8.0	—	3+	2+	1+	P
11.0	7.7	2+	3+	1+	P
13.0	7.7	3+	2+	2+	T
13.6	20.0	2+	2+	1+	A
17.0	6.1	4+	1+	1+	P
38.5	24.3	4+	1+	2+	T
42.0	—	4+	1+	1+	P
66.0	8.7	3+	0+	1+	A

* A = Absent; P = Present; T = Tumor.

frog Ringer solutions containing added potassium chloride were applied to frog sartorius preparations, the effects produced were similar to those obtained with thymus, spleen and striated muscle extracts. Low concentrations of added potassium (1.28 to 12.8 mEq L.) gradually reduced tension response of test muscle to indirect and direct stimulation. The activity of these solutions roughly paralled activity of thymus extracts containing comparable concentrations of potassium. A T_{50} of 2.9 minutes was obtained when a thymus extract containing 40 mEq/L. of potassium was tested whereas a T_{50} of 4.5 minutes was obtained when a frog Ringer solution containing 40 mEq/L of added potassium was tested. The time course of recovery of the tension curve after washing with frog Ringer was similar to that observed after tissue extracts had been tested on the sartorius preparation. Addition of $CaCl_2$ to thymus extracts (5 to 10 times normal concentration of $CaCl_2$ in frog Ringer) decreased the inhibitory effects of the extracts on the tension curve of frog sartorii. In a typical experiment, the T_{50} of the acetone insoluble fraction was 2.6 minutes whereas after addition of $CaCl_2$ (total concentration—5 x 10^{-3} M) the T_{50} increased to 8.5 minutes.

Thymus Gland Pathology. In 15 glands tested, 9 showed hyperplasia and germinal center formation, 4 failed to show these changes and 2 were thymomas. Table III shows that thymus glands with the most parenchyma possessed the greatest inhibitory activity.

Discussion. The original intention of repeating previously published experiments concerning the muscle inhibiting effects of thymus extracts (Wilson, Obrist, and Wilson) (5) as a point of departure for isolation of the active material led to investigation of potassium content of acetone-extracts of thymus tissue. The finding of a heat stable, dialysable substance in thymus extracts and extracts of other tissues which rendered the test nerve-muscle preparation inexcitable on indirect and direct stimulation suggested that potassium might be responsible for the observed inhibitory action. Presence of potassium concentrations ranging from 2 to 44 times normal potassium content of frog Ringer confirms the idea that potassium alone could be responsible for the inhibition observed in our experiments and in experiments reported by others (Wilson, Obrist and Wilson) (5). Furthermore, correlation between potassium concentration of thymus and other extracts and their ability to depress tension curve of the frog sartorius preparation strongly suggests that this is the case. Duplication of these effects by adding equivalent concentrations of KCl to frog Ringer solutions constitutes additional evidence that potassium concentrations which occurred in thymus extracts can produce rapid decrease of muscle tension. Failure of activity of acetone-soluble fraction to correlate well with potassium content is probably due to the protective effect of lipids in the test solution.

The deleterious effect of increased potassium concentrations on muscle excitability has long been recognized(8-10). According to Gellhorn's data(10) at the sodium concen-

tration used in our experiments (0.134 M), concentrations of potassium chloride in excess of 96 x 10^{-4} M produce rapid loss of excitability. Table II shows that the thymus extracts tested contained 48 to 660 x 10^{-4} M of potassium. Gellhorn(10) showed that calcium ions also antagonized the inhibitory effect of increased potassium concentrations. Approximately equal amounts of $CaCl_2$ above the concentration (8.1 x 10^{-4} M) of $CaCl_2$ in frog Ringer antagonized approximately equal amounts of KCl added to normal frog Ringer solution. Thus, in experiments where $CaCl_2$ was added to thymus extracts, the decrease in inhibitory activity of the extract can be attributed to antagonism between high concentrations of potassium in the extracts and the added calcium ions.

It should be emphasized that the concentrated tissue extracts used contain other ions (Na^+, Ca^{++}, Mg^{++}) in undetermined concentrations which, because of abnormal ion ratios, may also contribute to inhibitory action of these extracts on isolated frog nerve muscle preparation. Therefore, we cannot conclude that elevated potassium concentrations alone are responsible for the inhibition observed. Furthermore, we cannot conclude that extracts of myasthenic thymus glands prepared in this way are unique in containing considerable quantities of potassium. There is no evidence to indicate that myasthenic thymus glands contain more tissue potassium than normal thymus glands of comparable cellularity. Since the extracts tested in this study were prepared according to the methods of Wilson and associates(5), it is probable that the results reported by these investigators may be partly, if not entirely, due to high concentration of potassium in the extracts. The combined acetone soluble and acetone insoluble extracts used by Wilson and associates may have contained considerable concentrations of potassium.

It is of interest that in our study a rough correlation was found between cellularity of thymus glands tested, their potassium content and the activity of the extracts. Extracts prepared from glands which contained little fatty and fibrous tissue contained more extractable potassium and possessed greater activity. Thus, it might be expected that a myasthenic or a normal child's thymus might contain more inhibitory activity than the fatty, fibrous glands of adults as reported by Wilson and associates(5). However, it is not possible to explain on this basis the positive correlation between extract activity and the post-operative course of the patients reported by these investigators. The contractures observed by Wilson and associates(5) when high concentrations of thymus extracts were added to frog rectus abdominis muscle preparations may have been due to potassium in the extracts(11-13). The potentiation of acetylcholine action on the frog rectus preparation by thymus extracts reported by Wilson and associates(5) may also be due to potassium ions in the extracts.

Since simple acetone and saline extraction of tissue breis results in crude mixtures of many components including small ions in varying proportions, extreme caution must be exercised in interpreting the effects produced by these mixtures on nerve-muscle preparations. Of particular importance is potassium which in low concentrations blocks the neuromuscular junction (Overton)(14) and in high concentrations abolishes the excitability of the muscle.

It is possible that the inhibitory activity of myasthenic thymus extracts may not entirely be due to potassium in the extracts, but inhibitory activity not due to potassium cannot be determined in the presence of excessive potassium concentrations or abnormal concentrations of other ions in the test solutions. Although desalting procedures might eliminate this source of error, it is probable that other extraction methods should be used in searching for inhibitory activity in thymus glands from patients with myasthenia gravis.

Summary. 1. A method is described which permits recording of the isolated myogram of indirectly or directly stimulated isolated frog sartorius muscle. 2. Acetone extracts prepared from thymus glands obtained from 16 myasthenic patients, one normal child's thymus, 3 spleens and 2 samples of striated muscle were tested on the isolated frog sartorius

preparation. 3. Both acetone soluble and acetone insoluble fractions markedly decrease the muscle tension curve in 2 to 20 minutes. When the muscle becomes inexcitable on indirect stimulation, it is also inexcitable on direct stimulation. 4. The active material is heat stable, dialysable and is antagonized by $CaCl_2$ added to the extracts. 5. Concentrations of potassium 2 to 44 times the potassium concentration of frog Ringer were found in the extracts. These concentrations of potassium are sufficient to produce the muscle inhibition observed. 6. The significance of these results relevant to the search for a blocking agent in the thymus glands of myasthenic patients is discussed.

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1. McEachern, D., *Med.*, 1943, v22, 1.
2. Schweitzer, A., *J. Exp. Med. and Surg.*, 1947, v5, 264.
3. Constant, G., Porter, E. L., Andronis, A., and Rider, J. A., *Texas Rep. Biol. and Med.*, 1949, v7, 350.
4. Constant, G. A., Porter, E. L., Seybold, H. M., and Andronis, A., *Am. J. Physiol.*, 1949, v159, 565.
5. Wilson, A., Obrist, A. R., and Wilson, H., *Lancet*, 1953, v1, 368.
6. Wilson, A., and Stoner, H. B., *Q. J. Med.*, 1944, v13, 1.
7. Castleman, B., and Norris, E. H., *Medicine*, 1944, v38, 27.
8. Sereni, E., *J. Physiol.*, 1925, v60, 1.
9. Fenn, W. O., and Cobb, D. M., *J. Gen. Physiol.*, 1934, v17, 629.
10. Gellhorn, E., *Biol. Bull.*, 1930, v59, 338.
11. Solandt, D. Y., *J. Physiol.*, 1935, v84, 3.
12. Raventos, J., *Q. J. Exp. Physiol.*, 1938, v27, 99.
13. Torda, C., and Wolff, H. G., *J. Lab. and Clin. Med.*, 1947, v32, 1374.
14. Overton, E., *Pflug. Arch.*, 1904, v105, 176.

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Serum Complement in Hepatobiliary Diseases.* (22111)

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There is considerable suggestive evidence in the literature that the liver may be involved in the formation of serum complement and that the latter is diminished in liver disease and in experimental hepatic injury. Reports prior to 1929 were reviewed by Goldner(1). They indicate diminution of serum complement (C′) in experimental phosphorus or alcohol poisoning and its disappearance following hepatectomy in rabbits. In perfusion experiments with heat-inactivated serum, guinea pig liver contributed complement to perfusing serum, enabling the latter to hemolyze sheep red cells. On the other hand, complement remained normal in abdominally eviscerated dogs which had retained their livers. Goldner, using a rabbit hemolysin-sheep cell system in which *complete* hemolysis was used for the C′ titer, found that C′ was subnormal in 8 of 20 cases of "icterus catarrhalis" and in 10 of 11 cases of (cholangitic and portal) cirrhosis; furthermore, 3 of 7 cases of metastatic tumor of the liver showed diminution or absence of complement, one case of acute yellow atrophy complete disappearance of C′, while 6 cases of cholecystitis revealed no change from normal. Recently, Jordan(2), using his hematocrit method, reported marked diminution of C′ in all of 18 cases of cirrhosis studied; 22 cases of hepatitis showed some drop in C′ near the peak of the disease, while 12 patients with obstructive jaundice yielded high values. In contrast to these findings, Dulaney(3), using a 50%-hemolysis endpoint as determined pho-

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TABLE I. Serum Complement in 65 Patients with Hepatobiliary Disease.

Diagnosis	No. of patients	Age of patients, yr Range	Age of patients, yr Mean	Serum bilirubin, mg/100 ml Range	Serum bilirubin, mg/100 ml Mean	Serum complement, units Range	Serum complement, units Mean
Acute hepatitis							
Virus A or B	16 (3)	13–60	30	1.1–31.2	14.7	1.4–5.7	3.2
Leptospiral	3	37–52	44	12.0–22.8	18.3	3.8–4.1	3.9
Toxic	1		22		38.8		1.2
Cirrhosis							
Alcoholic	18 (12)	25–56	40	.4–27.0	5.3	1.2–4.7	2.8
Posthepatitic	2	47, 75		3.5, 9.6		.8, .8	
Biliary obstruction							
Benign	9*	28–60	48	.8–28.0	9.9	2.0–6.1	4.0
Malignant	16†	39–72	57	.8–34.0	9.6	1.9–4.9	3.5

( ) denotes presence of complicating conditions.
* Acute (2 cases), chronic cholecystitis (4), common duct stone (3).
† Carcinoma of gastrointestinal tract (4 cases), pancreas (9), biliary tract (2) and kidney (1), causing jaundice and/or metastatic hepatomegaly.

tometrically, detected no significant changes in C′ titer in 25 patients with liver disease, including 5 cases of infectious hepatitis and 12 of cirrhosis. However, in the course of the same investigation, this author found C′ diminished in each of 24 patients with induced malaria.

In an attempt to clarify this apparent conflict in the literature, the present study was undertaken.

Materials and methods. Collection of all blood specimens was done in Atlanta† from subjects in pre-prandial state. Only those patients were included whose diagnoses appeared established either by autopsy, biopsy, surgical operation, or prolonged clinical observation. Twenty healthy persons served as normal controls, 65 patients had various forms of liver or biliary tract diseases (Table I), and 25 patients had miscellaneous illnesses. These included pneumonia (3 cases), acute rheumatic fever (5), infectious mononucleosis (1), aseptic meningitis (1), chronic pancreatitis (3), rheumatoid arthritis (1), sickle cell anemia (2), sarcoidosis (1), lymphoma (1), multiple myeloma (1), myocardial infarct (1), malignant hypertension (1), disseminated lupus (1) and acute (1) and burned out stage of glomerulonephritis (2). Promptly upon separation from the clots, the sera were stored at –40°C and, packed with dry ice, were transported by air to New York for analysis (K.L.), by the method previously reported(4). Fifty % hemolysis was used as endpoint. A serum which produced 50% hemolysis in the same dilution as a standard 1:8 guinea pig serum was said to contain 1 unit of C′. Obviously then, a serum which produced 50% hemolysis at twice the dilution of the standard serum contained 2 units, etc. In our experience the simultaneous testing of a standard guinea pig serum in each test represents an indispensable control over many variables, especially the changing fragility of the sheep cells.

Results. The serum complement ranged in the normal control group from 1.5 to 4.4 units, with a mean of 2.7. Corresponding figures for the abnormal control group, exclusive of one case of acute glomerulonephritis and one of disseminated lupus, were 1.4-4.7, and 2.9. The C′ titer of the latter 2 cases was severely diminished to 0.1 and 0.5 unit, respectively. In previous studies involving 166 control cases, one of the authors (K.L.) had found a normal range of 1.1 to 3 units with an average of 1.7(5). Hence, for classification of the present results, 1.1 units was taken as the lower limit of normal and 1.1 to 1.4 units were considered as borderline range. Two distinctly subnormal values in the hepatobiliary disease group (Table I) were obtained in the 2 patients with posthepatitic cirrhosis, 1 and 2 weeks, respec-

† Grady Memorial Hospital, Emory University Hospital and Vet. Adm. Hospital.

tively, before their deaths from hepatic insufficiency. Three other cases of uncomplicated portal cirrhosis with ascites and low serum proteins had borderline titers. C′ titers in all other cases tabulated were either within or above the normal range. While these values represent initial examinations upon admission to the hospital, the majority of these patients had one or more follow-up determinations; in no instance was a subnormal value encountered.

Discussion. It is a well accepted fact that inflammation may result in a rise of C′ (4,6,10-13). This fact seems to be borne out by the cases of benign obstructive jaundice, many of whom had an accompanying cholecystitis and who showed the highest mean C′ values, as well as by the majority of the hepatitis patients, who showed elevated C′ values. From the data presented, it appears that hepatocellular disease does not lower the C′ titer. The slight diminution of C′ in the 2 cases of posthepatitic cirrhosis should probably be rather attributed to the moribund state of these patients than to the presence of hepatic disease. A drop in C′ associated with extremely grave conditions, including overwhelming infections has been observed by other authors(1,3,7).

The discrepancy between our data and those of Goldner(1) and of Jordan(2) cannot be readily resolved, but may be justifiably attributed to differences in analytical technics. Certainly, the method used in this study could be trusted to reveal evidence of subnormal C′ levels, which it has uncovered with great consistency in acute and subacute glomerulonephritis, in nephrosis and in disseminated lupus erythematosus(4,5).

The observed absence of a C′ drop in hepatitis would suggest that there is no widespread antigen-antibody reaction taking place in this disease, in contrast to glomerular diseases and to hypersensitivity states, in which such a reaction has been postulated(4,5,6-9).

The lack of a C′ diminution in hepatocellular disease speaks against formation of C′—or, at least, its limiting component—in or through the liver‡ and tends to shift attention to the reticulo-endothelial system as its physiologic source. In contradistinction to Jordan(2), who speaks of "the great discriminatory power of the complement test," our data do not indicate that this offers any aid in the differential diagnosis of liver disease, with the exception, perhaps, of a grave prognosis in the occasional event of C′ diminution.

Summary. The serum complement titer was determined in 20 patients with acute hepatitis, 20 with hepatic cirrhosis, and 25 with (benign and malignant) biliary obstruction. The only distinctly subnormal values were encountered in 2 moribund patients suffering from cirrhosis. The data speak against formation of complement in or through the liver and fail to indicate usefulness of complement titration in diagnosis of hepatobiliary diseases.

‡ Authors found C′ elevated in 2 patients in whom about two-thirds of the liver had been surgically removed because of hepatoma.

1. Goldner, M., *Dtsch. Med. Wchschr.*, 1929, v55, 390.

2. Jordan, F. L. J., *Acta Med. Scandinav.*, 1953, v144, 268.

3. Dulaney, A. D., *J. Clin. Invest.*, 1948, v27, 320.

4. Lange, K., Graig, F., Oberman, J., Slobody, L., Ogur, G., and LoCasto, F., *Arch. Int. Med.*, 1951, v88, 433.

5. Lange, K., Slobody, L., and Strang, R., *Pediatrics*, 1955, v15, 156.

6. Wedgewood, R. J. P., and Janeway, C. A., *ibid*, 1953, v11, 569.

7. Fischel, E. E., and Gajdusek, D. C., *Am. J. Med.*, 1952, v12, 190.

8. Kellett, C. E., *Lancet*, 1952, v2, 911.

9. Stavitsky, A. B., *J. Imm.*, 1952, v69, 63.

10. Hadjopoulos, L. G., *J. Lab. and Clin. Med.*, 1928, v14, 131.

11. Ecker, E. E., Seifter, S., Dozois, T. F., and Barr, L., *J. Clin. Invest.*, 1946, v25, 800.

12. Fischel, E. E., Pauli, Ruth H., and Lesh, J., *ibid.*, 1949, v28, 1172.

13. Vaughan, J. H., Bayles, T. B., and Favour, C. B., *Am. J. Med. Sc.*, 1951, v222, 186.

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Interaction of 16 Epi-Estriol and Estradiol 17β on Uterine Growth.*† (22112)

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In a previous publication the authors reported on estrogenic activity of 16 epi-estriol, an estrogen recently extracted from urine of pregnant women by Marrian and Bauld(1). Our results showed conclusively that this estrogen was slightly less active than estriol (2). Since estriol was shown to have the ability to interfere with uterine growth-promoting action of estradiol 17β(3), it seemed of considerable interest to determine the effects of 16 epi-estriol on estradiol 17β-induced uterine growth.

Therefore, the present study takes for its central theme a series of experiments designed to elucidate the effects of 16 epi-estriol on uterine growth-promoting and vaginal cornifying ability of estradiol 17β.

Procedures. Virgin, albino rats, approximately 100 days of age, were used. They were started on treatment 7 days after bilateral ovariectomy. The 16 epi-estriol was dissolved in U.S.P. propylene glycol, and estradiol 17β was dissolved in sesame oil and/or propylene glycol. They were injected subcutaneously, each of these compounds at a separate site, daily for 3 days. Vaginal smears were taken daily, and 72 hours after first injection necropsies were performed. Uteri were removed quickly and slit longitudinally on bibulous paper, thus removing excess uterine luminal fluid. The uteri were then weighed to nearest 0.2 mg on a Roller-Smith torsion balance. After wet weights were determined, the uteri were placed in an oven at 110°C for dry weight determinations. A standard period of 24 hours at 110°C was used uniformly for drying each uterus. After this period the weight of each uterus was determined on a Voland Analytical Balance.

Results. These results clearly indicate that 5 μg of 16 epi-estriol are required to demonstrably restrict in part the uterine growth promoting action of 0.1 μg estradiol 17β on both a wet and a dry weight basis. Smaller doses of 16 epiestriol when given in combination with our standard dose of 0.1 μg estradiol 17β do not seem to appreciably modify the response of the uterus to the more potent estrogen (Table I). On the other hand it seems quite apparent that a marked reduction in uterine growth is effected when 10 μg or more of 16 epi-estriol are administered concurrently, but at different injection sites, with the estradiol 17β (Table I). Actually, a maximal re-

TABLE I. Influence of 16 Epi-estriol on Uterine Growth Promoting Effects of Estradiol 17β in Ovariectomized Rats.

Treatment	No. of animals	Uterine wet wt, mg	Uterine dry wt, mg
Ovariectomized controls, no treatment	25	115.5 ± 3.3†	22.4 ± .7
.1 μg E*	42	227.3 ± 6.1	42.3 ± 1.8
1.0 μg 16 epi + .1 μg E	21	214.2 ± 6.5	39.5 ± 4.7
2.0 μg 16 epi + .1 μg E	7	203.7 ±11.1	37.6 ± 3.2
5.0 μg 16 epi + .1 μg E	7	190.5 ± 9.5	35.1 ± 2.6
8.0 μg 16 epi + .1 μg E	13	191.4 ± 7.3	35.0 ± 2.2
10.0 μg 16 epi + .1 μg E	6	174.9 ± 6.5	34.9 ± 1.0
25.0 μg 16 epi + .1 μg E	7	173.3 ± 8.8	33.7 ± 2.1
Reference controls: 16 epi alone			
1.0 μg	8	150.0 ± 8.6	26.1 ± .8
5.0 "	8	142.7 ± 6.8	25.8 ± 1.7
10.0 "	9	171.4 ± 8.7	28.5 ± 1.6
25.0 "	9	164.3 ± 5.1	30.5 ± 1.1

* E = Estradiol 17β; epi = epi-estriol.
† Avg ± stand. error.

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striction of uterine growth is obtained on the combined treatment of 0.1 μg estradiol 17β plus 10 μg 16 epi-estriol. Of particular significance is the fact that a summation of the two estrogens does not obtain, even though the maximal growth potential of the uterus is at least 50% more than that reported for 0.1 μg estradiol 17β(3).

This substance does not seem to alter the vaginal cornifying effects of estradiol 17β. The vaginal smears of those animals on 0.1 μg estradiol 17β with and without 16-epi-estriol seem to be relatively indistinguishable, both showing a preponderance of cornified epithelial cells with degenerative nuclei. Thus, the restrictive action of 16 epi-estriol seems to be mainly on the uterine growth-promoting effects of estradiol 17β.

These results quite clearly parallel those of Hisaw, Velardo, and Goolsby(3), who reported that estriol likewise inhibited estradiol 17β on the growth of the uterus. Furthermore, Huggins and Jensen(4) showed that 16 epi-estriol was quite efficacious in restricting the effects of estrone on the uterus in hypophysectomized rats. Therefore, these results seem wholly consonant with the thought that the various estrogens and/or their metabolites act *in concert* to produce the end organ result (3). These experiments provide additional data that give credence to the concept that an endocrine response is accomplished through a precise ratio of the hormone and/or its metabolites.

Summary. The adult, ovariectomized rat was used to ascertain the effects of a new estrogen, 16 epi-estriol, on the activity of estradiol 17β. These results indicate that when 3 daily doses of 5 to 25 μg of 16 epi-estriol are subcutaneously injected into rats receiving 0.1 μg estradiol 17β daily for the 3-day period, the response of the uterus to estradiol is restricted. Normally, 3 daily doses of 0.1 μg estradiol 17β increase the uterine wet weight from the value of the ovariectomized rat from approximately 116 mg to 227 mg. The simultaneous administration of 5, 10, or 25 μg of 16 epi-estriol in addition to the estradiol treatment reduces the response to 191 down to 173 mg. The inhibitory action is likewise reflected in the dry weight data. No apparent modification of the vaginal cornifying action of estradiol could be induced by 16 epi-estriol.

1. Marrian, G. F., and Bauld, W. S., *Biochem. J.*, 1955, v59, 136.

2. Velardo, J. T., and Sturgis, S. H., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 181.

3. Hisaw, F. L., Velardo, J. T., and Goolsby, C. M., *J. Clin. Endo. and Metab.*, 1954, v14, 1134.

4. Huggins, C., and Jensen, E. V., *Abst. Nat. Acad. Sci. in Science*, 1955, v121, 622.

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Hemolysis of Sheep Erythrocytes Sensitized with Leptospiral Extracts. (22113)

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Serodiagnosis of leptospirosis presently is based on agglutination, agglutination-lysis (AL), and complement-fixation procedures. The agglutination test employing killed leptospirae has been found to parallel the AL test in its type specificity(1). Gochenour(1) found that complement-fixation tests employing sonically-disrupted leptospiral antigens(2) showed a broader spectrum than AL procedures. Various modifications of AL procedure, such as that of Stoenner(3), may alleviate some difficulties associated with AL technic, in this case the hazard of working with living leptospirae. However, several serotypes of leptospirae known to be involved in leptospirosis make it necessary to employ several antigens in the above technics used as screening tests, the choice of serotypes depending on specificity of procedure and the purpose of the investigation(4). The need

for screening procedure employing antigen with specificity sufficiently broad to manifest antibodies produced against all serotypes of leptospirae would seem apparent. Recent progress was made by Chang and McComb (5), who described a hemagglutination (HA) procedure using human blood group O erythrocytes. Their HA test exhibited fairly broad specificity, based upon titers which were, however, very moderate in comparison with those of the highly type-specific AL reaction. Several reports(6-10) appeared concerning microbial substances capable of sensitizing erythrocytes in the presence of homologous antiserum to lytic action of complement (C′). Preliminary studies in this laboratory demonstrated that ethanol extracts of leptospirae were capable of sensitizing sheep erythrocytes to subsequent agglutination by homologous rabbit antisera, confirming the work of Chang (5) using human group O cells. Further investigation showed that sensitized sheep erythrocytes were susceptible to lysis in the presence of high dilutions of homologous rabbit antisera and complement.

This report describes the performance of the hemolytic test (HL) and results of studies to ascertain its specificity and sensitivity in comparison with the AL and HA procedures.

Materials and methods. Cultures—*L. pomona, L. icterohemorrhagiae B, L. bataviae, L. grippo-typhosa, L. hebdomadis,* and *L. canicola B* were obtained from Dr. Harold S. Bryan, College of Veterinary Medicine, University of Illinois. *L. autumnalis, L. icterohemorrhagiae C, L. canicola C* were obtained from Dr. R. S. Chang, Harvard University School of Public Health. *L. canicola R, L. hyos, L. ballum,* and *L. pyrogenes* were obtained from the Veterinary Division, Army Medical Service Graduate School. All cultures were grown in a medium consisting of 0.2% sterile tryptose-phosphate broth (Difco). Before use, sterile rabbit serum was added to final concentration of 10% and final medium heated in 56°C waterbath for 30 minutes. This medium was simple to prepare and yielded leptospiral growth comparable with other contemporary leptospiral media. *Diluent.* Phosphate-buffered isotonic saline was used in AL and HA procedures. It consisted of 0.137 M NaCl, 0.01 M Na_2HPO_4, and 0.003 M KH_2PO_4, yielding pH 7.2. Veronal-buffered saline was prepared as described by Mayer(11) and used as diluent in the HL procedure. *The AL Procedure* followed that of Gochenour(1), except that sera were decimally diluted. *Immune sera* were obtained from rabbits injected with viable 5 to 7-day-old cultures of leptospirae. Before use in HA and HL tests, each serum was inactivated and adsorbed with 0.1 volume of washed sheep erythrocytes. Pre-inoculation sera were collected from each rabbit, and checked for presence of leptospiral antibodies by all 3 procedures. *Leptospiral extracts* were prepared essentially as reported by Chang(5), with the exception that the 90% ethanol precipitate was washed in absolute ethanol, dried, and weighed. Dried, weighed extract was then redissolved in merthiolated saline to final stock concentration of 0.2 mg/ml. In later experiments, incorporation of veronal diluent at pH 7.4 in stock solution maintained extracts at constant activity in the HL test for several months. One fraction has been so maintained with constant activity for over 8 months. Active extracts were prepared from all leptospiral cultures excepting *L. grippo-typhosa* and *L. ballum,* from which extracts have been prepared with at most only slight HL activity. Considerable difference occurred between HL activities of extracts obtained from different culture growths of the same organism. *Sensitization of Erythrocytes for HA and HL Procedures.* One volume of 10% suspension of washed sheep erythrocytes was mixed well with 10 volumes of appropriate dilution of leptospiral extract. Erythrocyte suspension and fraction dilution were prepared in appropriate diluents. The mixture was agitated intermittently for 1 hour in 37°C waterbath, centrifuged, and the packed erythrocytes washed once with diluent. The sensitized erythrocytes were then reconstituted to a 1% suspension in appropriate diluent. *HA Procedure.* One-tenth ml volumes of sensitized erythrocytes were added to 0.4 ml volumes of decimal dilutions of sera. The cell control contained sensitized

TABLE I. Reciprocal Agglutination-Lysis Titrations.*

Leptospiral antigens	Anti-leptospiral rabbit sera											
	autumnalis	*ballum*	*bataviae*	*canicola B*	*canicola R*	*grippo-typh.*	*hebdomadis*	*hyos*	*ictero B*	*ictero C*	*pomona*	*pyrogenes*
autumnalis	4	1	—	—	—	1	2	—	—	4	2	—
ballum	—	4	—	2	2	—	—	—	1	—	—	4
bataviae	—	—	5	—	—	—	2	1	1	—	—	—
canicola B	—	2	—	4	5	1	1	—	2	—	—	2
canicola C	1	2	—	4	5	2	1	—	3	1	—	2
canicola R	—	2	—	4	5	—	—	—	3	—	—	3
grippo-typh.	—	—	—	—	—	4	1	—	1	2	1	—
hebdomadis	1	—	—	—	—	—	5	—	—	—	—	—
hyos	—	—	2	1	—	1	1	5	—	—	—	—
ictero B	1	2	—	3	3	—	—	—	4	—	—	3
ictero C	3	—	—	—	1	—	1	—	1	5	2	—
pomona	2	—	—	—	—	—	—	—	—	2	4	—
pyrogenes	—	4	—	2	2	1	—	1	1	—	—	5

* Titers expressed as logs of highest decimal dilutions showing definite reaction. "—" indicates no reaction in a dilution of 10^{-1} or higher.

erythrocytes and diluent. Serum control contained lowest dilution of serum and non-sensitized erythrocytes as a check on adsorption. Tubes were agitated and incubated at 30°C for 16-20 hours, and read by gently flicking the tube. *HL Procedure.* One-tenth ml volumes of sensitized erythrocytes were added to 0.4 ml volumes of decimal dilutions of sera. Two exact units of guinea pig C′ in 0.2 ml volume were added to each tube, followed by addition of 0.3 ml of diluent. Serum control contained non-sensitized in place of sensitized erythrocytes. C′ control contained diluent in place of serum, and sensitized cell control contained sensitized erythrocytes plus 0.9 ml of diluent. Tubes were agitated and incubated 1 hour in 37°C waterbath and read for hemolysis in usual manner, recording hemolysis as complete, partial, and negative. *Standardization of Leptospiral Extracts for HA and HL Procedures.* Double dilutions of the extract starting from 0.2 mg/ml were used to sensitize erythrocytes in the HA procedure. Such sensitized erythrocytes were then set up against master dilutions of known homologous immune rabbit sera in HA and HL procedures. Titration of all dilutions of extract against each serum dilution in HL tests was made in presence of 0.2 ml of 1:30 dilution of guinea pig C′ per tube, which was consistently found to represent an excess of C′. The end-point or concentration of extract used in further tests was selected as highest dilution, or smallest amount, above which there was no decrease in activity. Acceptable levels of extract activity demonstrated homologous immune serum HA titers between 1:10 and 1:100 and HL titers of 1:100 to 1:10000. Satisfactory HA and HL activity was usually achieved with extract concentrations ranging from 2.0 to 200 mg/ml. Some extracts in high concentrations hemolyzed erythrocytes in diluent; however, the end-point was always several dilutions higher than that which caused hemolysis. *Titration of C′ for Use in HL Procedure.* Using leptospiral extract which had been diluted according to previous standardization and the usual decimal dilutions of homologous immune serum, series of titrations were set up, each with increasing 0.05 ml increments of guinea pig C′ diluted 1:30. Volumes of C′ ranged from 0.05 to 0.3 ml. The end-point or 1 exact unit of C′ was taken as the smallest amount showing complete lysis in highest dilution of immune rabbit serum, and 2 exact units in 0.2 ml volume were used thereafter in HL procedure.

Results. Reciprocal AL titrations between various leptospiral serotypes and their homologous and heterologous antisera were carried out. It is apparent from Table I

TABLE II. Reciprocal Hemolytic Titrations.*

Leptospiral antigens	Anti-leptospiral rabbit sera											
	autumnalis	*ballum*	*bataviae*	*canicola B*	*canicola R*	*grippo-typh.*	*hebdomadis*	*hyos*	*ictero B*	*ictero C*	*pomona*	*pyrogenes*
autumnalis	3	3	4	3	3	2	4	4	3	2	3	3
bataviae	2	3	3	2	2	2	2	3	2	2	2	2
canicola B	2	3	2	2	2	2	2	3	2	2	2	2
canicola C	3	4	4	3	4	3	4	4	3	3	3	4
canicola R	3	3	3	3	3	2	3	4	3	3	3	3
hebdomadis	2	2	2	2	2	2	2	2	2	2	2	2
hyos	3	3	3	2	3	2	3	3	3	2	2	2
ictero B	3	4	3	3	3	2	3	4	3	3	3	3
ictero C	3	3	4	4	4	3	4	4	4	4	3	4
pomona	3	4	5	4	4	3	4	5	4	5	3	4
pyrogenes	2	2	2	2	2	2	2	2	2	1	2	2

* Titers expressed as logs of highest decimal dilutions showing definite reaction.

that AL reaction is quite type-specific as is generally recognized. The same master dilutions of immune rabbit sera were used to set up similar reciprocal HL titrations (Table II). HL reaction is extremely group specific. Antibodies produced against any one of the leptospiral types reacted broadly with all leptospiral fractions studied. The finding of higher heterologous than homologous titers with some antisera is felt to reflect differences in activity between fractions obtained from different leptospiral types. For example, highly active fractions were obtained consistently from our cultures of *L. pomona, L. icterohemorrhagiae,* and *L. canicola C.* However, as mentioned previously, only the slightest activity was obtained from extracts prepared from *L. ballum* and *L. grippo-typhosa.* Fractions obtained from *L. pyrogenes* were consistently low in HL activity. On the basis of results in Tables II and III, *L. pomona* was selected as the culture from which we obtained extracts for use in screening human sera for leptospiral antibodies by HL and HA procedures.

Selected leptospiral extracts were used to sensitize sheep erythrocytes which were then cross-titrated in HA procedure with homologous and heterologous immune rabbit sera (Table III). Although only selected extracts were used in HA titrations, it should be mentioned that, as in HL titration, extracts prepared from *L. ballum, L. grippo-typhosa,* and *L. pyrogenes* consistently possessed minimal activity or were completely inactive. The HA reaction is apparently similar in spectrum to the hemolytic reaction; however, HA titers were consistently lower, which causes the degree of crossing to appear less significant than in HL procedure.

TABLE III. Reciprocal Hemagglutination Titrations.*

Leptospiral antigens	Anti-leptospiral rabbit sera											
	autumnalis	*ballum*	*bataviae*	*canicola B*	*canicola R*	*grippo-typh.*	*hebdomadis*	*hyos*	*ictero B*	*ictero C*	*pomona*	*pyrogenes*
hebdomadis	2	2	1	2	2	1	2	2	2	1	2	2
canicola C	2	2	2	2	2	1	2	2	2	1	2	2
canicola R	1	1	1	1	1	1	1	1	1	—	1	1
ictero C	2	2	2	2	2	1	2	2	2	1	2	2
pomona	2	2	2	2	2	1	2	2	2	1	2	2

* Titers expressed as logs of highest decimal dilutions showing definite reaction. "—" indicates no reaction in a dilution of 10^{-1} or higher.

TABLE IV. Comparative Titrations on Human Sera.

No. of sera showing result		Result using following titrations: AL	HL	HA
505		—*	—	—
9		+†	—	—
10		—	+	—
23		—	—	+
1		+	+	—
24		—	+	+
0		+	—	+
8		+	+	+
Total sera	580			
Total positive	75	18	43	55
% positive	12.9%	3.1%	7.4%	9.5%

* Negative = No reaction in dilution of 10^{-1} or higher.
† Positive = Complete reaction in dilution of 10^{-1} or higher.

To compare relative activity of each of the 3 procedures, AL, HA, and HL titrations were performed on 580 human sera submitted at random from hospitals and clinics in this area (Table IV). With none of the patients was an initial clinical diagnosis of leptospirosis considered. All 3 procedures were performed as previously described, and the AL test was performed using 3 types: *L. pomona, L. ictero B,* and *L. canicola B.* It should be mentioned that of the 580 sera, only 2 febrile patients were available for repeat serum titrations. One patient showed progressively increasing titers to both AL and HL procedures to peak at 1:10000 levels. The AL titers were against *L. icterohemorrhagiae* and *L. canicola,* and the HA titer remained at 1:10 throughout. The other patient never showed a positive AL titer with any of the 3 leptospirae used as antigens; however, the HL titer progressively increased during course of the illness to a level of 1:10000, and the HA titer rose to a level of 1:100. Six of the positive sera showed AL titers of 1:1000 or higher, and 3 showed AL titers of 1:10000 or higher. In a similar fashion 21 sera showed HL titers of 1:1000 or higher and 5 were as high as 1:10000. None of the sera showed HA titers above 1:100.

It should be noted that the apparent advantage of the HA procedure in detecting antibodies missed by the other two procedures (23 sera) was based upon an HA reaction in serum dilutions of only 1:10, without which the positive HA reactions would drop to 1.1%. The reason for this number of weak HA reactions and their connection with leptospirosis is not known. The sera had been adequately adsorbed with sheep erythrocytes, as evidenced by negative controls. In contrast, positive reactions in AL and HL procedures were based predominantly upon complete reactions in serum dilutions of 1:100 to 1:10000.

Ability of HL procedure to detect antibodies in titers of 1:100 or higher missed by AL procedure could be readily explained on the basis of differences in specificity of the reactions. Thus, it is felt that a total of 31 of the 34 sera in Table IV may be showing high HL titers stimulated by serotypes of leptospira other than the 3 used in these AL tests. Ability of the AL test to detect antibodies missed by the other 2 procedures (9 sera) is at present unexplained; however, it is possible that this reflects differences in persistence of antibody levels detectable by the 3 procedures. Clarification of this point would depend upon the difficult task of coupling similar studies on selected human cases of leptospirosis with adequate cultural studies, extending over many months from very early in the course of the illness.

If leptospiral extracts should prove to remain stable over long periods of time with respect to HL activity by other workers, it would seem that the HL procedure would show promise as a convenient and useful tool with which to screen human sera for leptospiral antibodies. Although it requires an extra step in the addition of complement over the HA procedure, it is as easily read, more rapid in performance, and considerably more sensitive. It is certainly less hazardous to the operator than the AL procedure. HL procedure would seem to warrant further study as a screening tool by those laboratories where adequate bacteriological control studies as well as patient followups would be available.

Summary. Leptospiral extracts are capable of sensitizing sheep erythrocytes to the HL action of homologous and heterologous lepto-

spiral antiserum and complement, and description of the HL procedure is presented. The HL procedure was shown to be broadly specific, in sharp contrast to the high degree of type specificity seen in the AL procedure. In comparison with HL titrations, the HA reaction was also found to have broad specificity but to be considerably less sensitive than the HL reaction. All 3 procedures were compared in a study involving 580 randomly selected human sera, from which data it would seem that the HL procedure warrants further study as a possible screening tool for the rapid detection and titration of leptospiral antibodies in human sera.

1. Gochenour, W. S., *Symposium on the Leptospiroses*, Army Medical Service Graduate School, 1953, 160.

2. Randall, R., Wetmore, P. W., and Warner, A. R., *J. Lab. Clin. Med.*, 1949, v34, 1411.

3. Stoenner, H. G., *Am. J. Med. Res.*, 1954, v15, 434.

4. Wolff, J. W., *Laboratory Diagnosis of Leptospirosis*, American Lecture Series, Chas. C. Thomas Pub., 1954.

5. Chang, R. Shih-man, and McComb, D. E., *Am. J. Trop. Med. Hyg.*, 1954, v3, 481.

6. Hayes, L., *Aust. J. Exp. Biol. and Med. Sci.*, 1951, v29, 51.

7. Neter, E., Bertram, L. F., Zak, D. A., Murdock, M. R., and Arbesman, C. E., *J. Exp. Med.*, 1952, v96, 1.

8. Fisher, S., *Aust. J. Exp. Biol. Med. Sci.*, 1950, v28, 613.

9. Middlebrook, G., *J. Clin. Invest.*, 1950, v29, 1480.

10. Cox, Charles D., Zerschling, Anne, and Meloy, Robert, PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 693.

11. Mayer, M. M., *et al.*, *J. Exp. Med.*, 1948, v88. 427.

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Failure of Synthetic 5-Hydroxytryptamine Creatinine Sulfate to Enhance Clot Retraction of Platelet-Poor Human Plasma.* (22114)

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Many platelet constituents with specific effects on the hemostatic mechanism have been recently identified(1). Whether they represent intimate constituents of platelets or are carried by them still requires investigation. These agents include a thromboplastic factor (2), constituents accelerating conversion of prothrombin to thrombin and of fibrinogen to fibrin(3), an antiheparin factor(4), possibly antifibrinolysin(5,6). An additional substance, 5-hydroxytryptamine (serotonin), has been identified in dried platelets preparation (7,8) and shown to liberate from platelets during clotting of human blood(9). Serotonin has been shown to possess vasoconstrictor and capillary protecting activity(10). Recently, it has also been suggested that serotonin is able to induce clot retraction of platelet-poor bovine and human plasma(11,12). This observation is of considerable importance since clot retraction has been generally considered to be due to intact platelets rather than to a single platelet constituent. This study is an attempt to evaluate the clot retraction promoting effect of the commercially available synthetic derivative of serotonin, 5-hydroxy-tryptamine creatinine sulfate, the use of which is currently recommended in the treatment of the bleeding tendency of thrombocytopenic states. At the same time, observations have been made on the ability of this compound to influence the rate and extent of prothrombin conversion during clotting of platelet-poor plasma.

Materials. Native platelet-poor plasma.

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TABLE I. Effect of 5-Hydroxy-tryptamine Creatinine Sulfate on Clot Retraction and Prothrombin Utilization during Clotting of Undiluted Platelet-Poor Human Plasma.*

Final conc. of 5-hydroxy-tryptamine creatinine sulfate, mg %	Clot retraction, % (24 hr after clotting)	Prothrombin activity of serum, % (1 hr after clotting)
80	10.5	87
40	10	84
20	9.5	86
10	10	92
5	8	84
2.5	8.5	84
1.25	9	93
.6	9.5	93
.3	10	87
.1	10	90
Control†	8	90

* Avg results of 4 experiments.
† Saline solution, 1/10 volume.

Blood was collected from fasting healthy donors with the "double syringe technic" in Silicone-coated syringes through Arquad 2-C coated needles. Blood was transferred to pre-cooled Silicone-coated test tubes and centrifuged in the cold (4°C) at 3,000 for 30 minutes. The upper ⅔ of supernatant plasma only were then promptly transferred to another pre-cooled Silicone-coated test tube and promptly added to the various dilutions of the substance under study. Silicone-coated pipettes were used in all steps. *Citrated platelet-poor plasma.* Blood was collected with the same technic and transferred to Silicone-coated test tubes containing 1/10 volume of 0.2 M solution of sodium citrate. Blood and anticoagulant were promptly mixed by inverting the tube which had been stoppered with paraffin-coated cork. *Synthetic 5-hydroxy-tryptamine creatinine sulfate,* was made available through the courtesy of Dr. Merrill E. Speeter.§ Serial dilutions were made in saline solution, and strengths used are indicated in Tables I and II. *Determination of clot retraction.* Immediately after completion of clotting, clot was rimmed by the technic of pushing a thin glass rod to the bottom and circling it around the clot. Tubes were then incubated in water bath at 37°C for 1 or 3 hours. After visual inspection, volume of serum expressed when the tube was inverted at an angle of 60° was taken as measure of retraction. *Serum prothrombin time (prothrombin consumption)* was determined by our method(13), 1 or 3 hours after completion of clotting.

Results. 1. *Effect of 5-hydroxy-tryptamine creatinine sulfate on clot retraction and prothrombin utilization during clotting of platelet-poor native human plasma.* Experiments were made in duplicate. Various concentrations of 5-hydroxy-tryptamine creatinine sulfate in a constant volume of 0.1 ml were transferred to chemically clean glass test tubes kept in water bath at 37°C; 0.9 ml of native platelet-poor human plasma were then added to each tube and the contents mixed. A similar experiment was performed using Silicone-coated test tubes and comparable results were obtained. There was no appreciable and constant effect on the speed of clotting, clot retraction and serum prothrombin activity one and 3 hours respectively after completion of coagulation. There was no appreciable difference in clot retraction 24 hours after clotting between tubes containing 5-hydroxy-tryptamine creatinine sulfate and the control containing 1/10 volume of saline solution, as shown in Table I.

2. *Effect of 5-hydroxy-tryptamine creatinine sulfate on clot retraction of diluted native or citrated human plasma.* Native or citrated human plasma were diluted 1:5 with sterile 0.85% NaCl solution. 4.5 ml of diluted plasma were added to a series of glass test tubes kept in water bath at 37°C con-

TABLE II. Effect of 5-Hydroxy-tryptamine Creatinine Sulfate on Clot Retraction and Prothrombin Utilization during Clotting of Diluted Platelet-Poor Human Plasma.*

Final conc. of 5-hydroxy-tryptamine creatinine sulfate, mg %	Clot retraction, % (24 hr after clotting)	
	Citrated plasma†	Native plasma
40	74	
20	72	[illegible]
10	70	
Control‡	72	

* Avg of several experiments.
† Clotted with bovine thrombin.
‡ Containing saline solution, 1/10 volume.

§ Director of Biochemical Research, Upjohn Co.

taining various concentrations of 5-hydroxy-tryptamine creatinine sulfate in a constant volume of 0.5 ml. Diluted native plasma was allowed to clot spontaneously. Clotting often did not occur in some instances when plasma had been made practically platelet-free by centrifugation. Diluted citrated plasma was clotted with the addition of 0.2 ml saline solution containing 60 units of purified bovine thrombin. Clot retraction was observed and volume of separated serum measured 3 and 24 hours after completion of coagulation. Results 24 hours after clotting are presented in Table II. Residual prothrombin activity was negligible because of the high dilution of plasma. There was no demonstrable effect on retraction of the clot.

Discussion. Correll *et al.*(10) studied the thromboplastic activity of serotonin derivatives by investigating their ability to accelerate the clotting time of plasma. Their negative results are confirmed by ours. On the other hand. our results on effect of serotonin derivatives on the retraction of the clot are not confirmatory of those already presented in the literature(11,12). Since, however, it is not known whether synthetic products and 5-hydroxy-tryptamine carried or liberated by platelets are identical in structure and properties, our negative experiment does not prove that platelet serotonin may not contribute to the mechanism of clot retraction, as postulated by Fenichel and Seegers.

Perhaps, explanations might be offered for the discrepancy between our findings and those of other authors. There may be differences in purity of the reagents used. Moreover, the complexity and multiplicity of the factors influencing clot retraction has been emphasized by many authors and recently summarized by Budtz-Olsen(14). Thus, it is likely that, without extremely careful control of all experimental conditions, results may be at great variance and not constantly reproducible. Since little is known of the ideal conditions under which clot retracts *in vitro* and *in vivo,* the evaluation of the clot promoting activity of a drug remains a hazardous problem.

Summary. Synthetic 5-hydroxy-tryptamine creatinine sulfate failed to enhance clot retraction and prothrombin utilization during clotting of platelet-free human plasma. These results are at variance with recently reported findings.

1. Stefanini, M., *Am. J. Med.,* 1953, v14, 68.

2. Stefanini, M., and Campbell, E., *Revue d'Hémat.,* 1954, v9, 576.

3. Ware, A. G., Fahey, J. C., and Seegers, W. H., *Am. J. Physiol.,* 1948, v154, 140.

4. Van Creveld, S., and Paulssen, M. M. P., *Lancet,* 1952, v1, 23.

5. Johnson, S. A., and Schneider, Ch. L., *Science,* 1953, v117, 229.

6. Stefanini, M., and Murphy, I. S., *J. Clin. Invest.,* in press.

7. Bracco, M., and Curti, P. C., *Haematologica,* 1953, v37, 721.

8. Zucker, M. B., Friedman, B., and Rapport, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 82.

9. Zucker, M. B., *Int. Physiol. Congress,* 1953, v19, 915.

10. Correll, J., Lyth, L., Long, S., and Vanderpoel, J., *Am. J. Physiol.,* 1952, v163, 537.

11. Fenichel, R. L., and Seegers, W. H., *ibid.,* 1955, v181, 19.

12. Ballerini, G., *Atti Acc. Sc. Ferrara,* 1954-55, v32, 1.

13. Stefanini, M., and Crosby, W. H., *Blood,* 1950, v5, 964.

14. Budtz-Olsen, O. E., Clot retraction. Charles C. Thomas, Springfield, 1951.

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Delayed Blood Coagulation in Rabbits with Localized Shwartzman Reaction. (22115)

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In previous work(1) the authors elaborated a procedure indicating the presence or absence of fibrinogen in serum derived from minimally heparinized blood. Subsequently, a correlation between the utilization of fibrinogen and the rheumatic state was attempted. The results seemed to indicate an accelerated fibrinogen polymerization in active rheumatic disease. In order to obtain experimental verification of the reported clinical findings this procedure, described as "fibrinogen polymerization test"(2), was applied to rabbits in which a Shwartzman phenomenon had been produced. Minimally heparinized rabbit blood withdrawn 24 hours after the provocative intravenous injection of endotoxin failed, however, to coagulate within an observation period of 7 or 8 hours so that the test could not be carried out properly, since gross coagulation of the heparinized specimen is a prerequisite. This observation seemed to indicate delayed coagulation independent of the *in vitro* addition of minimal amounts of heparin. Consequently, it was postulated that the Shwartzman phenomenon may be associated with a prolonged Lee-White clotting time. Verification of this assumption was sought in the experiments reported herein.

Materials and methods. Twenty rabbits of either sex, each weighing approximately 2 kg, received 0.5 cc of meningococcus endotoxin* intradermally, followed by the intravenous administration of 2.0 cc of endotoxin 24 hours later. On the 3rd, 4th, 5th and 8th day of the experiment, Lee-White clotting times were determined with blood withdrawn by cardiac puncture. About 15 cc were swiftly aspirated after careful shaving of the precordium and palpating of the point of maximum cardiac impulse. Only a portion of the aspirated 15 cc were utilized for the Lee-White clotting time, thus leaving a considerable amount of blood within the syringe assumed to contain most of the tissue juice with its thromboplastic properties. Three glass tubes measuring 14 mm x 10 cm were utilized for the determination of the Lee-White clotting time. About 2 cc of blood were placed into each tube. A fourth, graduated centrifuge tube containing 0.2 cc of 0.1 molar solution of sodium citrate was filled up to the 2 cc mark and served for the determination of prothrombin time according to the photo-electric modification of the one-stage Quick method(3). Lee-White clotting times were obtained at room temperature which varied from 22°-26°. The end point at which the clotting time was read coincided with the formation of a solid clot which permitted the complete inversion of the test tube without displacement of the coagulum.

Results. Typical localized Shwartzman reactions were elicited in 16 of the 20 rabbits employed in this study. Characteristic hemorrhagic necrotic lesions at the site of the sensitizing intradermal injection of the endotoxin appeared well before 24 hours had elapsed after the provocative intravenous administration of the meningococcal endotoxin. As seen from Table I, the mean Lee-White clotting time in all 20 animals was 18 minutes on the third day of the experiment, *i.e.* 24 hours

TABLE I. Mean Lee-White Clotting Times and Prothrombin Times of Rabbit Blood Obtained through Cardiac Puncture at Various Stages of Shwartzman Reaction.

	Days after initiation of experiment			
	3rd	4th	5th	8th
Lee-White clotting time in min.	18	8.3	7.4	4.3
Prothrombin time in sec	6.7	4.5	5.7	7.4

* We wish to express our appreciation to Dr. Gregory Shwartzman for providing meningococcus endotoxin for the experiments reported herein.

after the provocative intravenous injection, at which time the Shwartzman reaction was quite pronounced and declined to 8.3, 7.4 and 4.3 minutes on the 4th, 5th and 8th day. No group distinction could be established between the clotting times of the majority of rabbits in which the Shwartzman reaction was visible and the few animals in whom no necrotizing lesion appeared. In a separate group of 10 normal rabbits the clotting time of intracardiac blood was determined at room temperature and found to have a mean value of 5.5 minutes.

Prothrombin times in all animals subjected to the Shwartzman phenomenon appeared to be normal throughout (mean value 6.1 seconds) and did not disclose any definite lengthening, even at the time when the Lee-White clotting times were markedly prolonged.

Discussion. Although no satisfactory explanation of the pathogenesis of the Shwartzman phenomenon has yet been found(4), it is known to be characterized by a hemorrhagic necrosis in the prepared skin site. It seems that the second-provocative-injection of bacterial filtrate leads to injury of the walls of small blood vessels. Moreover, thrombosis of these vessels has been found to be a constant pathological feature(4-6).

The results of our study disclose that the localized Shwartzman reaction is accompanied by a prolongation of the Lee-White clotting time with a mean value of 18 minutes as compared to a normal mean of 5.5 minutes. Although the nature of this prolonged clotting time remains undetermined at present, the release of heparin or heparinoid substances as tissue reaction to anaphylactoid stimuli(7) seems to offer an explanation. The prolonged clotting time may contribute to the hemorrhagic nature of the Shwartzman reaction. On the other hand, it is not of such magnitude as to prevent the phenomenon as has been achieved with large doses of heparin(8,9), namely 30 mg per kg producing incoagulability of rabbit blood for about 7 hours(6). Nevertheless, it seems to be of interest that rabbit blood which physiologically displays a high degree of hypercoagulability should lose this property when the localized Shwartzman phenomenon is induced. Hypercoagulability of rabbit blood is manifested not only in a relatively short Lee-White clotting time, but also in a rather short prothrombin time (Table I).

Summary. 1. A localized Shwartzman reaction was produced in 20 rabbits. 2. The Lee-White clotting time determined with blood obtained through cardiac puncture was found considerably prolonged when the reaction appeared and gradually returned to normal during the ensuing 6 days.

1. Losner, S., and Volk, B. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 417.

2. ———, *Am. J. Med. Sc.*, 1955, v229, 371.

3. Losner, S., Volk, B. W., Jacobi, M., and Newhouse, S., *J. Lab. and Clin. Med.*, 1950, v36, 473.

4. Shwartzman, G., Phenomenon of Local Tissue Reactivity. Paul B. Hoeber, New York, 1937.

5. Gerber, I. E., *Arch. Path.*, 1936, v21, 776.

6. Stetson, C. A., *J. Exp. Med.*, 1951, v93, 489.

7. Ungar, G., and Damgaard, E., *ibid.*, 1955, v101, 1.

8. Cluff, L. E., and Berthrong, M., *Bull. Johns Hopkins Hosp.*, 1953, v92, 353.

9. Good, R. A., and Thomas, L., *J. Exp. Med.*, 1953, v97, 871.

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Hepatic Damage in Rats Fed Human Foodstuffs.* (22116)

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It is well established that laboratory animals fed one or another deficient diet may develop hepatic lesions resembling Laennec's cirrhosis. These observations have supported the view that an inadequate dietary intake contributes to the development of cirrhosis in human subjects. By and large, however, the experimental diets have had little resemblance to the diets eaten by such patients. The hypothesis that dietary deficiency is important in the pathogenesis of human cirrhosis would be strengthened if similar lesions appeared in animals fed foodstuffs resembling those eaten by patients with this disease. Gillman, Gillman, Mandelstam and Gilbert(1) were able to produce severe hepatic damage in rats fed corn meal and sour milk, a diet similar to that eaten by South African natives who commonly develop disease of the liver. The changes observed in rats included diffuse fatty liver, cirrhosis, and atrophy or hypertrophy of individual hepatic lobes without parenchymal necrosis. It seemed of interest to determine whether experimental animals would develop hepatic changes if fed diets similar to those of patients with cirrhosis of the liver in this country. The experiments to be described suggest that rats fed such diets may develop lesions which bear a similarity to those of human cirrhosis.

Method. Young male albino rats of the Wistar strain obtained from Carworth Farms, New City, N. Y., were housed in individual cages with open meshed bottoms. All rats were fed Purina dog chow and tap water *ad libitum* until the onset of the experimental period, at which time their mean weight was about 110 g. The rats were then fed one of 4 diets *ad libitum* as follows: Diet A (12 rats) —Purina dog chow and tap water, Diet B (45 rats)—spaghetti, olive oil and wine; the wine was diluted with an equal volume of water, Diet C (12 rats)—spaghetti, olive oil and 10% glucose in water, Diet D (12 rats) —spaghetti, olive oil and diluted wine with vitamin supplement. The composition of these diets is given in detail below. In 10 rats fed diet B and in all those fed diets C and D the amount ingested was measured grossly, taking into account spillage. For mechanical reasons the measurement of spillage was only approximate. As a result only a crude estimate of the actual dietary intake was possible. The animals did not have access to their feces or urine. Purina dog chow is composed of meat meal, dried skimmed milk, wheat germ, dried beet pulp, corn grits, cereal feed from corn and wheat, dried fig, soy bean oil meal, molasses, riboflavin supplement, brewer's dried yeast, Vit. A and D feeding oils, one per cent steamed bone meal, and one per cent iodized salt. According to the manufacturer, an average sample contains 23.0% protein, 6.0% fat, 9.6% ash, and 6.0% crude fiber. In addition, an average sample contains carotene, 9 mg, thiamin, 5.6 mg, and riboflavin, 5.0 mg per 100 g. Procino-Rossi brand elbow spaghetti is made of "high grade A-No. 1 semolina." Analysis by the Wisconsin Alumni Research Foundation demonstrated that 100 g of this spaghetti contained 10.9% moisture, 0.65% ash, 0.17% ether-extractable material, 13.38% protein, 0.24% crude fiber and 74.66% carbohydrate. There were 2.25 mg of cystine, 1.0 mg of methionine and 1.84 mg of choline per gram of spaghetti. This spaghetti is not fortified with vitamins. Analysis of the Parma California Burgundy was kindly provided by Dr. Aram Ohanesian, chief chemist of Cella Vineyards. One hundred ml contained 13.2% alcohol (by volume), 0.74 g of acid (as tar-

* This work would not have been possible without the assistance of Miss Joan E. Colopy, Mrs. Velma H. Axelrod and Mrs. Rosemary Shaft.

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TABLE I. Influence of Diet on Development of Hepatic Lesions in Rats.

Diet	Total animals	Rats sacrificed when free of symptoms	Rats which died or sacrificed when moribund	Hepatic lesions
A. Purina dog chow & water	12	12 at 158 to 343 days	0	Normal liver
B. Spaghetti, olive oil & wine	45	6 at 45 to 223 days	39 at 13 to 223 days	Necrosis, fatty infiltration and fibrosis
C. Spaghetti, olive oil & 10% glucose in water	12	4 at 139 to 343 days	8 at 191 to 273 days	Fatty infiltration and fibrosis
D. Spaghetti, olive oil, wine & vitamins	12	10 at 139 or 343 days	2 at 271 and 343 days	Normal liver

taric acid), 0.14 g of reducing sugar, 0.19 g of tannins and 0.025 g of protein. The pH of the undiluted wine was 3.65. The wine was diluted with an equal volume of water and was fed in water bottles. No analysis was available of the olive oil used, but pure olive oil is said to be composed entirely of lipids (2) and is devoid of choline(3). Olive oil is notably rich in unsaturated fats; the oil used at no time appeared to be rancid. The vitamin supplement in Diet D consisted of 1 ml of an aqueous suspension containing 125 μg of thiamine, 100 μg of riboflavin, 125 μg of pyridoxine and 750 μg of calcium pantothenate per ml added to each 100 ml of diluted wine. In addition, one drop of cod liver oil USP was added to the spaghetti once weekly.

Results. The effect of the various diets tested is summarized in Table I. Diet A (Purina dog chow and tap water). These animals grow rapidly and remained healthy in appearance and behavior. All were sacrificed while in good condition from 158 to 343 days after the onset of the experimental period. Autopsy showed no significant abnormality. Histologically the liver and kidney were normal in every instance.

Diet B (spaghetti, olive oil, and wine). The rats gained weight at a progressively diminishing rate for about 30 days and then gradually lost weight until they died or were sacrificed. Six rats were sacrificed without obvious symptoms from 45 to 223 days after the start of the experiment, while 39 died or were sacrificed when moribund at periods ranging from 18 to 223 days.

Seven of the 45 animals showed severe diffuse hepatic necrosis. This lesion was noted in 4 of the 12 rats which died within 115 days of the start of the experiment and in only 3 of the 33 rats which survived longer than this. The necrosis involved all lobules and affected small groups of cells. The latter were usually shrunken and presented acidophilic homogenous cytoplasm with indistinct borders, while the nuclei were reduced in size, pyknotic, and often fragmented. There was also focal coagulation necrosis with no nuclear content. The necrotic cell groups alternated with fairly well preserved hepatic cells, whose cytoplasm was vacuolated. Marked fatty degeneration was present in all the necrotic livers. No leukocytic reaction was evident. The liver cords were commonly fragmented and the sinusoids prominent. In a few animals there were foci of necrosis in which there was complete loss of liver architecture. These lesions were associated either with hemorrhage or hyperemia. There were no distinct nodular regenerative foci. There was slight to moderate fibrosis, bile duct proliferation, and lymphocytic exudate in the portal areas. However the fibrous reaction within the lobules was mainly of condensation type. In 8 additional rats on this diet there were slight to moderate grades of fatty degeneration or fatty infiltration, mainly periportal in location and in a few instances associated with a mild degree of fibrosis.

Diet C (spaghetti, olive oil, and 10% glucose in water). These rats gained a small amount of weight for a month or 2, and then their weight either remained relatively stationary or decreased slightly until they died or were sacrificed. Four animals in this group

were sacrificed without symptoms at 139 to 143 days and 8 died or were sacrificed in a moribund state at 139 to 273 days. In the rats in this group, parenchymal necrosis was not observed. However there were 5 animals with moderate or marked fatty infiltration of the liver, principally periportal, with formation of the signet-ring-type of hepatic cell. All were associated with a mild degree of periportal fibrosis.

Diet D (spaghetti, olive oil, wine, and vitamins). These rats either gained weight slowly but steadily or reached a maximum from which their weight did not decline, unlike the rats fed Diets B and C. Ten rats were sacrificed without symptoms at 139 to 343 days, one died at 271 days, and one was sacrificed when moribund at 343 days. There were no significant lesions of the liver in this group. No hepatic cell necrosis was found. There was one animal with a slight degree of periportal fat infiltration.

During the first 60-day period, at a time when none of the rats on measured diets had died or been sacrificed, a group of 10 rats fed diet B ate an average of 9.6 g of spaghetti and drank 14.1 ml of diluted wine per day; spillage prevented an accurate measure of the olive oil ingested. Rats offered Diet C averaged 7.9 g of spaghetti and 19.4 ml of glucose daily, and rats fed Diet D, 9.4 g of spaghetti and 10.8 ml of wine, as well as vitamin supplements. Thus rats eating a diet of spaghetti, olive oil and wine, with or without vitamin supplements, consumed 17 mg of choline, 18 mg of methionine, and 21 mg of cystine daily. Those fed a diet in which glucose was substituted for wine consumed 14 mg of choline, 15 mg of methionine, and 18 mg of cystine daily. It cannot be assumed, however, that the rats continued to ingest these quantities of food at a later time.

Discussion. Chronic alcoholism is common in patients with Laennec's cirrhosis of the liver in the United States. Nonetheless, it has not been possible to demonstrate that the administration of alcohol to experimental animals leads to hepatic lesions. Within recent years, attention has been drawn to the possible part played by chronic dietary de-

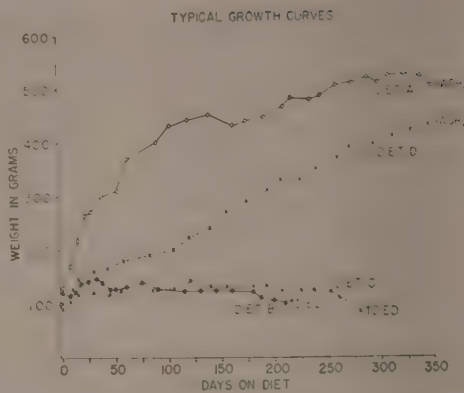

FIG. 1. Typical growth curves of rats fed Diets A, B, C and D.

ficiency in the pathogenesis of cirrhosis in chronic alcoholic patients. This view has been supported by the apparent beneficial effects of a nutritious diet in patients with this disease and by the production of hepatic lesions in experimental animals. In general, the lesions in experimental animals have been of two types, namely, fatty infiltration and fibrosis, prevented by choline or methionine, and necrosis of liver cells, prevented by cystine or methionine(4-6). However, the diets used in most experimental studies have been composed of foods not ordinarily eaten by human beings.

In the present experiments, rats were fed diets containing foods commonly used by man. These diets consisted of some of the foodstuffs consumed by patients with cirrhosis who are of Italian extraction, an ethnic group in which this disease occurs more commonly than in the rest of the population of the United States(7). In these experiments, both diffuse necrosis of liver cells and fatty infiltration and fibrosis were noted; the relative frequency of the lesion depending upon the nature of the diet and the duration of survival. Rats fed spaghetti, olive oil and wine (Diet B) developed necrosis of liver cells, fatty degeneration and fibrosis. Necrosis was relatively more frequent in those rats which died within the first 4 months of the experiment than in those which survived longer than this time. Rats fed spaghetti, olive oil and wine, supplemented by a vita-

min mixture not containing tocopherol (Diet D) had no significant lesions even though they did not appear to ingest more spaghetti than the rats fed the same diet without vitamins. The substitution of glucose for wine, without the addition of vitamins (Diet C) somewhat prolonged the time of survival, and at the same time modified the nature of the lesion so that fatty infiltration and fibrosis developed rather than necrosis. It is noteworthy that the glucose-fed rats obtained relatively more of their calories from glucose and less from spaghetti, when compared to wine-fed rats. The spaghetti was the sole source of choline and methionine.

Summary. Significant hepatic lesions, consisting of diffuse parenchymal necrosis and marked fatty infiltration, have been produced in rats fed diets of spaghetti, olive oil and wine or glucose solution. Both lesions were associated with a moderate degree of fibrosis. The specific dietary factors responsible for the changes were not ascertained.

1. Gillman, J., Gillman, T., Mandelstam, J., and Gilbert, C., *Brit. J. Exp. Path.*, 1945, v26, 67.

2. Bridges, M. A., *Dietetics for the Clinician*, 4th Edition, 1941, Philadelphia, Lea and Febiger.

3. Fletcher, J. P., Best, C. H., and Solandt, O. M., *Biochem. J.*, 1935, v29, 2278.

4. Daft, E. S., Sebrell, W. H., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 1.

5. Himsworth, H. P., and Glynn, L. E., *Clin. Science*, 1944, v5, 93.

6. ———, *ibid.*, 1944, v5, 133.

7. Richardson, W., *New Engl. J. Med.*, 1938, v218, 257.

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Lipides Distribution and Phospholipide Turnover in Tissues of Rabbit Shortly After Growth Hormone. (22117)

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The concept that arteriosclerosis is a disease of metabolism has focused attention on endocrine control of lipide metabolism and their relationship to development of arterial degenerative lesions. It is increasingly evident that for elucidation of this relationship more information is required of the modes of action and nature of effects of hormones on lipide metabolism in different tissues. Certain adrenocortical steroids(1-3) and epinephrine (3-5) were reported to exert an influence on lipide distribution in tissues and development of aortic degenerative changes. There is ample evidence that growth hormone causes accelerated mobilization and catabolism of endogenous fat(6-10), but there is little information concerning the effect of growth hormone on lipide partition in tissues. Recently, Greenbaum and MacLean(7) studied distribution of lipides in plasma and liver of intact rats at intervals after treatment with growth hormone. Greatest changes occurred 6 hours after growth hormone when plasma and liver neutral fat and plasma phospholipide were significantly increased. The authors suggested that increased plasma phospholipide could be explained by accelerated synthesis of this lipide since Geschwind *et al.*(11) reported increased turnover of liver phospholipide in normal and hypophysectomized rats after 14 days or more of treatment with growth hormone. Mayer and Jones(12) reported a significant, but slight, increase in blood cholesterol of mice after 2 weeks treatment with growth hormone.

The present study was undertaken to determine concurrently lipide partition and phospholipide turnover in plasma, liver and aorta of intact rabbits a few hours after treatment with growth hormone. Knowledge of phospholipide-cholesterol-neutral fat relationships and phospholipide metabolism in the above tissues was a necessary preliminary to a study of the influence of growth hormone on lipide

TABLE I. Summarized Data of the Effect of Growth Hormone on Body and Organ Weights, Plasma Glucose, Liver Glycogen.

	Plasma glucose, mg %	Liver glycogen, g %	Liver wt, g/kg	Aorta wt, g	Body wt, kg
Controls (9)	118 ± 1.7*	3.44 ± .35	29.0 ± 1.2	.409 (.300-.472)†	2.4 (1.9-2.7)
G.H. (9)	132 ± 2.3	4.43 ± .19	35.5 ± 1.2	.404 (.346-.466)	2.4 (2.2-2.7)
P =	<.001	.02	.004	ns‡	ns

* Mean ± S.E. † Mean and range of values in group. ‡ ns = not significant.
"P" = Probability of difference between means occurring by chance alone.

metabolism in experimental atherosclerotic rabbits.

Materials and methods. New Zealand white female rabbits of pedigreed stock were given a rabbit pellet diet and tap water *ad libitum* for several weeks prior to day of experiment. Food and water were not available during experimental period. Control animals received a single subcutaneous injection of physiological saline (0.5 ml/kg body weight). Another group of rabbits received a single subcutaneous injection of 5 mg/kg of anterior pituitary purified growth hormone (Armour Co., Lot #M308)* in sterile saline 10 mg/ml. Immediately after injection of the placebo or growth hormone, 0.5 mc of radioactive phosphate (P-32)† was given intravenously. Sample of heart blood (4-5 ml) was obtained 3 hours after P-32 injection for determination of radioactive and chemical plasma acid-soluble phosphates(13). Six hours after initial hormone or saline injection a large sample (30-40 ml) of cardiac blood was obtained, the animal then killed with intravenously administered overdose of EVIPAL (n-methyl-cyclo-hexenyl-methylbarbituric acid). Whole liver and aorta were removed. A section of liver (approx. 0.5 g) was taken for determination of liver glycogen(14,15). Plasma glucose was determined by the method of Nelson(16). A section of liver (about 2 g) was placed in ice-cold 10% trichloracetic acid and used for determination of inorganic and organic acid-soluble phosphates(17). Following homogenization of the liver sample, neutralization of filtrate to phenol red, and addition of magnesia mixture the samples were kept in the refrigerator overnight to permit magnesium ammonium phosphate to settle (18). After centrifugation determinations were done for P-31 and P-32 on the supernatant (organic acid-soluble phosphates) and the acid-dissolved precipitate (inorganic acid-soluble phosphates). A large specimen of liver, the whole aorta, and a plasma aliquot of terminal blood sample were submitted to hot alcohol-ethyl ether extractions. Combined alcohol-ether extractions were evaporated under reduced pressure, and residue redissolved in petroleum ether (b.p. 30-60°C). Suitable aliquots of petroleum ether were used for chemical and radioactive determinations as follows: Total lipid was calculated from weight of residue after evaporation of petroleum ether and drying to constant weight. Lipide phosphorus was determined(13,19), and phospholipides were calculated from lipid-P values x 26. Total and free cholesterol were determined by the method of Schoenheimer and Sperry(20). Neutral fat was calculated by subtraction of phospholipide, free cholesterol, and cholesterol oleate concentrations from total fat value. For radioactivity determinations of phospholipide and acid-soluble phosphorus suitable aliquots were mounted on Whatman ash-free filter paper backed by aluminum foil, and samples allowed to evaporate. After covering samples with Scotch tape, they were counted with a Victoreen No. 1B85 Geiger tube for a minimum of 4000 counts.

Results. Inspection of the data in Table I shows that plasma glucose and liver glycogen concentrations, and total liver weight were moderately, but significantly, increased a few hours after growth hormone.

With the exception of liver ester choles-

* The growth hormone used was supplied through the generosity of Dr. Irby Bunding of Armour and Co.

† Obtained from Abbott Laboratories, Oak Ridge, Tenn. as sodium radio-phosphate, sterile solution.

TABLE II. Effect of Growth Hormone on Concentration of Lipide Fractions of Plasma, Liver and Aorta 6 Hours after Administration in Rabbits.

Groups	Total lipide	Phospho-lipide	Cholesterol Total	Cholesterol Free	Cholesterol Ester	Neutral fat
			Liver lipide partition†			
Controls (9)	5.01 ± .15*	3.76 ± .07	.27 ± .01	.24 ± .008	.03 ± .008	.96 ± .13
G.H. (9)	6.24 ± .26	3.27 ± .09	.24 ± .01	.20 ± .008	.04 ± .006	2.54 ± .28
P =	.001	<.001	.05	.002	ns	<.001
			Plasma lipide partition			
Controls	.323 ± .031	.093 ± .012	.071 ± .007	.025 ± .002	.046 ± .005	.125 ± .015
G.H.	.599 ± .034	.154 ± .007	.114 ± .011	.043 ± .004	.072 ± .008	.245 ± .019
P =	<.001	<.001	.003	.001	.015	<.001
			Aorta lipide partition			
Controls	8.68 ± .61	.99 ± .03	.18 ± .003	.15 ± .004	.03 ± .004	7.49 ± .61
G.H.	6.61 ± .61	.95 ± .05	.19 ± .008	.16 ± .01	.03 ± .007	5.70 ± .58
P =	.025	ns	ns	ns	ns	.045

* Mean ± S.E. † All values expressed as g/100 g wet tissue wt or per 100 ml plasma.
"P" = Probability of difference between means occurring by chance alone.

terol, concentrations of the lipide fractions in plasma and liver were significantly changed following administration of growth hormone (Table II). Concentrations of liver phospholipide and free cholesterol were significantly decreased, while neutral fat was significantly elevated. Concentrations of all plasma lipide fractions were significantly increased after treatment with growth hormone. With regard to the aorta lipide partition, only a significantly decreased neutral fat was found in the treated group which was mainly responsible for depression of the aorta total lipide level.

In Table III are presented the specific activities of phospholipide of plasma, liver and aorta, and specific activities of acid-soluble phosphates of liver and plasma. Liver phospholipide specific activity was significantly increased in growth hormone-treated animals. Plasma phospholipide specific activity in the experimental group was not different from controls. However, this value, *per se*, is not a valid index of plasma phospholipide turnover since there had occurred a significant increase in chemical concentration of this lipide fraction. Calculation of the amount of radioactive phospholipide per ccm of plasma (specific activity × concentration) showed that the actual amount of plasma phospholipide synthesized during experimental period was 250% greater in

TABLE III. Effect of Growth Hormone on Turnover of Lipide and Acid-Soluble Phosphorus of Liver, Plasma, and Aorta.

Groups	Liver phospholipide-P mg/100 g	Liver phospholipide-P S.A. × 10^3	Plasma phospholipide-P mg/100 ml	Plasma phospholipide-P S.A. × 10^3	Aorta phospholipide-P mg/100 g	Aorta phospholipide-P S.A. × 10^3
Controls (9)	148.7 ± 4.2*	9.5 ± .9	2.52 ± .5	3.5 ± .6	37.18 ± 2.7	5.6 ± .4
G.H. (9)	130.9 ± 2.7	13.1 ± 1.4	5.87 ± .6	3.8 ± .7	38.93 ± 2.2	6.9 ± .7
P =	ns	.05	<.01	ns†	ns	ns

	Plasma Acid-soluble phosphate mg/100 ml	Plasma Acid-soluble phosphate S.A. × 10^3	Liver Inorganic phosphate mg/100 g	Liver Inorganic phosphate S.A. × 10^3	Liver Organic acid-soluble phosphate mg/100 g	Liver Organic acid-soluble phosphate S.A. × 10^3
Controls	5.75 ± .3	87.8 ± 8.5	25.0 ± .9	143.2 ± 15.0	105.0 ± 2.4	46.0 ± 5.3
G.H.	5.67 ± .2	93.1 ± 11.6	23.5 ± 1.5	136.3 ± 12.4	107.0 ± 4.7	41.9 ± 3.5
P =	ns	ns	ns	ns	ns	ns

* Mean ± S.E. † Amount of radioactive phosphorus (S.A. × conc.) incorporated in plasma phospholipides of rabbits injected with growth hormone was significantly greater than controls (P = < 0.001).
S.A. is specific activity expressed as percentage of injected dose/mg of phosphorus.
"P" = Probability of difference between means occurring by chance alone.

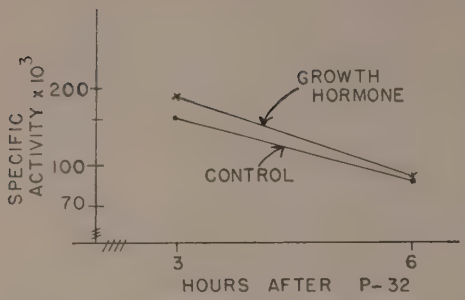

FIG. 1. Semi-logarithmic plot of the specific activities of plasma acid-soluble phosphate against time.

rabbits which received growth hormone than in controls. Inspection of terminal specific activities of acid-soluble phosphates of plasma and liver (Table III) and specific activity curves of plasma acid-soluble phosphate (Fig. 1) show that neither chemical concentration nor turnover of precursors of liver and plasma phospholipides were altered after growth hormone. It, therefore, may be concluded from the radioactivity data that accelerated formation of plasma and liver phospholipides occurred in treated rabbits. In contrast, the data show that specific activity of aortic phospholipide was not affected by growth hormone administration.

The magnitude of alterations (expressed as percentage change from controls) in various lipide fractions of plasma, and the entire liver and aorta of rabbits treated with growth hormone is shown in Fig. 2. Also shown for comparison are data recalculated from a recent experiment(5) illustrating changes in lipide fractions after a single subcutaneous injection of epinephrine in oil (1 mg/kg). Except for the different hormones administered, the conditions and experimental procedures in the two studies were exactly the same. It may be noted that in the group which received growth hormone, the greatest changes occurred in the neutral fat fraction of tissues analyzed: liver (+245%), plasma (+95%), and aorta (-25%). Also, liver ester cholesterol was changed +200%, while liver free cholesterol and phospholipide were considerably less (+25%). The plasma phospholipide, free and ester cholesterol were about 50% greater than control levels. On the other hand, 6 hours after epinephrine injection the changes determined in lipide fractions of plasma, liver and aorta were: liver neutral fat +217%, liver ester cholesterol +100%, and plasma phospholipide +25%. The extent of increases in the two liver lipide fractions after epinephrine was comparable to that found with growth hormone. In contrast with the alterations in lipide distribution produced by the latter hormone, it is apparent that the effects of epinephrine on lipide composition in these tissues was comparatively small.

Discussion. Our data showed that marked alterations in lipide distribution of plasma, liver and aorta, and significantly increased turnover of liver and plasma phospholipides occurred 6 hours after injection of growth hormone.

Changes in amounts and rate of synthesis of phospholipides of plasma and/or liver have been reported after exogenous administration of a variety of hormones and after destruction of function of certain endocrine gland(s). In studies in which blood cholesterol also was determined, a parallelism was noted in incre-

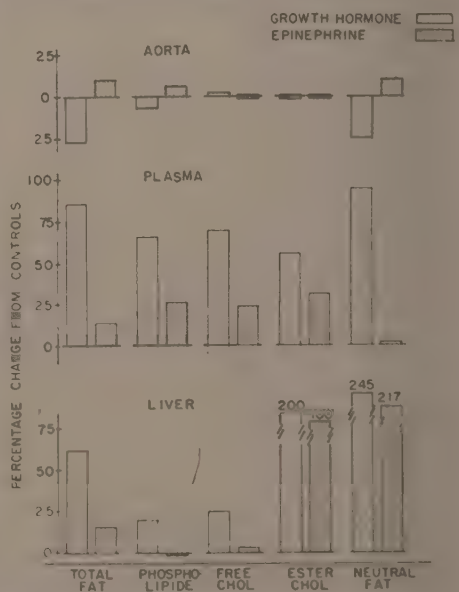

FIG. 2. Effect of growth hormone and epinephrine (see text for doses) on total lipide and lipide fractions in plasma and entire liver and aorta 6 hr after injection. Data are expressed as percentage change, increase or decrease, from respective control values.

ment or decrement of phospholipide and cholesterol levels(1-3,21). In this investigation also blood phospholipide and cholesterol were found elevated to a similar extent and accompanied by increases in the entire liver content of these two lipides. It is possible that related metabolic mechanism(s) are responsible for the regulation of blood levels of phospholipide and cholesterol; this control probably is hormonal. The radioactivity data indicated that accelerated synthesis of liver and plasma phospholipides occurred in animals administered growth hormone. Further, since practically all plasma phospholipide is believed to be produced in the liver the increased content of plasma phospholipide during the period of the experiment suggests an increased exchange of this lipide between liver and plasma had occurred. It does not seem feasible, however, at this time to deduce that alteration in rate of formation or amounts of phospholipides resulted from a specific action of growth hormone on this parameter of metabolism. This appears warranted in view of the variety of individual hormones and endocrine situations with which a change in phospholipide metabolism has been associated (22). Moreover, there is evidence(23) that changes in amount of phospholipide and their rate of formation in a given tissue are related to metabolic needs of the tissue, as an increased rate of fat catabolism. In considering this, Lotspeich and Petersen(9) and Campbell and Davidson(6) recently reported increased acetoacetate formation and oxygen uptake in liver slices obtained from rats a few hours after treatment with growth hormone. They interpreted these findings as indicating an accelerated catabolism of endogenous fat. Our data suggest that fat mobilization (accumulation of liver neutral fat and elevated plasma levels of this lipide) occurred concurrently with alterations in chemical concentrations and turnover of phospholipides of liver and blood. It, therefore, appears more likely, at least from the data now available, that increased amounts of phospholipide and their increased rate of formation a few hours after growth hormone, were not the resultant of a direct effect of this hormone on phospholipide metabolism but rather alterations in the latter concomitant but subsidiary to the primary action of growth hormone of accelerated mobilization and catabolism of endogenous fat. It is noteworthy in this regard that Dury(5) recently reported evidence of markedly increased turnover of phospholipide of plasma, liver, and aorta in intact rabbits shortly after a single injection of epinephrine while there occurred only a moderate increase in liver neutral fat, and none in the plasma.

Change in aorta lipide distribution after growth hormone has not to our knowledge been reported. The depressed level of aortic neutral fat is especially intriguing since in other studies done at this laboratory(3-5,24) it was found that aorta lipide distribution was not altered after acute or chronic exhibition of cortisone or epinephrine. Speculation on this finding is rendered difficult in view of the paucity of information on arterial lipide metabolism, and especially the influence of hormones on the latter. Two points, however, are noteworthy as possibly related to this subject: 1) data in this and other studies have demonstrated that the aorta is particularly rich in neutral fat; and 2) aorta lipide composition was affected, at least qualitatively, as adipose tissue following administration of growth hormone.

The magnitude of alterations of lipide distribution in plasma and liver and rapidity of occurrence were remarkable. Calculation of the relative contribution of different fractions to increased total lipide in the entire liver after growth hormone revealed that neutral fat contributed 47%, phospholipide 15%, free and ester cholesterol each contributed 1.5%. With regard to total lipide increment in plasma, the relative contribution of neutral fat was 53%, phospholipide 19%, free cholesterol 6%, and ester cholesterol contributed 8%. The decreased level of aorta neutral fat was responsible entirely for the fall in aorta total lipide content. Lotspeich and Petersen (9) suggested that the liver fat mobilizing action of growth hormone might be a secondary phenomenon which occurred as a result of a primary depletion of glycogen. We did not find such a change in the intact rabbit. Indeed, in this study the liver glycogen and blood glucose concentrations were moderately,

but significantly, greater than the respective control values. Since the experiment of above authors and the present one are not exactly comparable (differences of species, alimentation, and hormone dosage) a feasible explanation cannot be made of the different effects observed on carbohydrate metabolism.

We are inclined to believe that alterations in lipides distribution and accelerated phospholipide metabolism, in this study, are best explained by the concept proposed by Weil (10) of a primary adipokinetic action of growth hormone. Since this hormone apparently modifies lipide metabolism without mediation of other target glands, further studies concerning the role of growth hormone, if any, in the patho-physiology of arteriosclerosis seem warranted.

Summary. 1. A study was made of the effects of a single injection of anterior pituitary purified growth hormone on distribution of lipides and turnover of phospholipides in plasma, liver and aorta a few hours after administration in intact rabbits. 2. Plasma glucose and liver glycogen concentration, and total liver weight were moderately, but significantly, increased in animals which received growth hormone. 3. Contents of all lipide fractions of plasma and entire liver were significantly increased in experimental animals. In the aorta, the only change was a significantly decreased neutral fat content. 4. Liver total lipide content was increased 64%, plasma total lipide was increased 85%, and aorta total lipide was decreased 25%. The relative contribution of each lipide fraction to percentage increased or decreased total lipide determined in these tissues was as follows: in liver, neutral fat contributed 47%, phospholipide 15%, and free and ester cholesterol 1.5% each; in plasma, neutral fat contributed 53%, phospholipide 19%, free cholesterol 6%, and esterified cholesterol 8%; in the aorta, neutral fat contributed 100%. 5. Radioactivity data showed that accelerated synthesis of liver and plasma phospholipides occurred after treatment with growth hormone; but aortic phospholipide turnover was not different from controls. 6. It is suggested that increased rate of formation of phospholipides of liver and plasma need not necessarily be a specific effect of growth hormone but a change in this parameter of metabolism subsidiary to enhanced mobilization of endogenous fat. It is further suggested that all elements of altered distribution of lipides in tissues analyzed could be explained in accordance with the concept of a primary adipokinetic action of growth hormone.

1. Stumpf, H. H., and Wilens, S. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 219.

2. Gordon, D., Kobernick, S. D., McMillan, G. C., and Duff, G. L., *J. Exp. Med.*, 1954, v99, 371.

3. Dury, A., and DiLuzio, N. R., *Am. J. Physiol.*, 1955, v182, 45.

4. Dury, A., and Moss, L. D., *J. Gerontol.*, 1954, v9, 287.

5. Dury, A., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 508.

6. Campbell, J., and Davidson, I. W. F., *J. Biol. Chem.*, 1951, v189, 35.

7. Greenbaum, A. L., and McLean, P., *Biochem. J.*, 1953, v54, 407.

8.———, *ibid.*, 1953, v54, 413.

9. Lotspeich, W. D., and Petersen, V. P., *Am. J. Physiol.*, 1954, v176, 232.

10. Weil, R., *A.M.A. Arch. Int. Med.*, 1955, v95, 739.

11. Geschwind, I. I., Li, C. H., and Evans, H. M., *Endocrinol.*, 1950, v47, 162.

12. Mayer, J., and Jones, A. K., *Am. J. Physiol.*, 1953, v175, 339.

13. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, v66, 375.

14. Good, C. A., Kramer, H., and Somogyi, M., *ibid.*, 1933, v100, 485.

15. Somogyi, M., *ibid.*, 1945, v160, 61.

16. Nelson, N., *ibid.*, 1944, v153, 375.

17. Zilversmit, D. B., and DiLuzio, N. R., *ibid.*, 1952, v194, 673.

18. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *ibid.*, 1948, v176, 193.

19. Youngsburg, G. E., and Youngsburg, M. V., *J. Lab. and Clin. Med.*, 1930, v16, 158.

20. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, v106, 745.

21. DiLuzio, N. R., Shore, M. L., and Zilversmit, D. B., *Metabolism, Clin. and Exp.*, 1954, v3, 424.

22. Zilversmit, D. B., *Ann. Rev. Biochem.*, 1955, v24, 157.

23. Artom, C., *A Symposium on Phosphorus Metabolism*, 1952, v2, 203, The Johns Hopkins Press, Baltimore.

24. Dury, A., *Am. J. Med. Sci.*, 1955, v230, 427.

Received October 17, 1955. P.S.E.B.M., 1955, v90.

Comparison of Extracts of Tubercle Bacilli and BCG as Immunizing Agents in Guinea Pigs.* (22118)

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Recent investigators studying biological properties of fractions of tubercle bacilli have for the most part used the mouse for *in vivo* studies. Bloch and Noll(1) reported that tuberculosis in black mice of Strain C57 is enhanced by administration of cord factor. Weiss and Dubos(2) report that a fraction extracted from *Mycobacterium tuberculosis* by absolute methanol is capable of eliciting in mice a state of increased resistance to infection.

Notwithstanding these contributions based on a mouse evaluation of biological properties of fractions and extracts of the tubercle bacillus, we wish to report studies carried out with guinea pigs for *in vivo* determinations of immunizing activity of extracts of mycobacteria. Accordingly, we present results of experiments concerning immunizing activity in guinea pigs of extracts of non-heat killed tubercle bacilli.

Methods and materials. Most fractionation studies reported at this time were made using *Mycobacterium tuberculosis var. hominis* NIH strain 199RB. This culture has been used since the inception of this research in 1946.‡ Studies of strains more recently isolated from sputum are currently being made in an attempt to find a culture possessing greater immunizing potency than does 199RB. Variations were made in procedure for growth and fractionation of cultures from one experiment to another, to evaluate critically the conditions necessary for isolation of the most potent fraction; however, procedures in general were as follows. Mass cultures were prepared on modified Long's medium(3) made up in 25 liter quantities and distributed into one-gallon jugs, each one containing 900 ml. The inoculum for 30-60 such cultures, usually a 2-week-old surface growth, was taken from one 900 ml culture flask. The cultures were subsequently incubated at 37°C for 3 to 12 weeks. After incubation, the culture fluid was withdrawn into 5 gallon bottles containing Cresylone§ and mineral oil (antifoam), autoclaved and discarded. The culture pellicle from harvested jugs was combined, by means of a vacuum system, into one container which was then completely filled with the first extracting solvent (usually ethanol) and allowed to stand 48 hours. This step can usually be considered the point at which the cells become nonviable. Following ethanol treatment, the residue was extracted 24-48 hours with ethanol-ether (50-50), methanol-chloroform (50-50), or chloroform.

Preliminary separation of the extracted components was occasionally made by cooling the methanol-chloroform solution first to 5°C and collecting a 5°C insoluble fraction. The filtrate from this separation was cooled to –20°C at which temperature a second insoluble residue was collected. The filtrate upon evaporation deposits a residue designated the –20°C soluble fraction. Further separations of total extracts or low temperature insoluble fractions were made by column chromatography. For experiments here reported, final separations were made on 50 mm diameter chromatographic columns packed with 85 g of Magnesol‖-Celite¶ and charged with 2 g of

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‡ Previous to Sept., 1953 this research was supported by Parke-Davis and Co. and was under supervision of Dr. W. J. Nungester, Department of Bacteriology, University of Michigan.

§ Cresylone- tri-cresol disinfectant of Parke-Davis and Co.

‖ Magnesol—Magnesium Silicate, Product of Westvaco Chemical Co., S. Charleston, W. Va.

¶ Celite—Filter Aid, Johns Manville Co.

the substance to be separated. The columns were developed as adsorption-elution chromatograms with the following series of solvents; petroleum-ether (60-68°C boiling range), petroleum ether-benzene (50-50) benzene, benzene-diethyl ether (50-50) and various mixtures of methanol and chloroform. Infrared spectra were recorded for all extracts and chromatograph-eluted components for purposes of identification of the substance and as a control on reproducibility of the fractionation (Randall *et al.* 4-7). Guinea pigs weighing approximately 500 g were vaccinated with the lipid fractions dissolved in a mixture of 9 parts mineral oil and one part Arlacel A.** Bacillary residues and protein extracts were emulsified from aqueous suspension in the same adjuvant according to the method of Salk(8). As one of the controls, BCG was routinely administered in aqueous suspension either from cultures grown 8 days on liquid Tween-albumin medium or from lyophilized vaccine kindly supplied by Tice Laboratories through the courtesy of Dr. Sol. R. Rosenthal. All animals were vaccinated subcutaneously in the inguinal region or over the sternum. The protocol for each evaluation of bacillary extracts *in vivo* was changed from time to time but generally guinea pigs were vaccinated with 0.1 mg of extract or 1.0 mg of BCG. Three weeks after the second vaccination test guinea pigs and a suitable number of controls, treated with adjuvant alone, were shaved and tuberculin tested with 0.0002 mg of PPD by intracutaneous route. The results were read at 24 and 48 hours, a positive reaction being at least 5 mm minimum diameter. In many instances, reactions were strong with a blanched, later necrotic, center. Following the tuberculin test, all groups were randomized and challenged. The 18-20 guinea pigs in each group were housed in 3 or 4 stainless-steel cages. For randomization, the 3 or 4 cages in any one experimental group were redistributed at random so that one cage would be found in the first quarter of the total number, a second cage in the second quarter of the total and so on. The code for the newly assigned cage numbers was sealed and not examined until post mortem examination of all groups had been completed. The following advantages were presumed to result from randomization. It would tend to minimize the possibility that variations in challenge suspension or procedure would affect any one experimental group, and would also reduce unintentional bias which might be shown in evaluating the extent of gross disease at autopsy. During post mortem examination, each successive series of 5 animals has come from a different treatment group. This results in a constant change in extent of gross tuberculosis and would discourage any inadvertent attempt to reduce the variation from one group to the next.

The challenge suspension was prepared from an 8-day culture of strain 199RB on liquid Tween-albumin medium. The culture was first filtered through Whatman #1 filter paper, adjusted to 85% transmission at 500 μ (Coleman Junior spectrophotometer) and then diluted to 10^{-5} with saline containing 0.5% bovine albumin fraction V. Plate counts made of this challenge suspension contained 300-1000 viable units. The challenge was given subcutaneously in 1 ml of diluent in the inguinal region or over the sternum. Six weeks after challenge the guinea pigs were sacrificed (ether) and the extent of gross tuberculosis in the viscera estimated according to the scheme of Feldman(9). It is important to state that gross evaluation of the lungs was difficult to make in early experiments because of intercurrent infection with group "C" streptococcus, however it has been our experience with strain 199RB that the spleen is a good index of the extent of tuberculosis in the guinea pig. In order for this to be true, the challenge should be such that some animals will have minimal or no gross disease. If the spleen is extensively involved, showing enlargement and confluent tubercles, one expects to find disease in the lungs and liver. Conversely, if there is no splenic involvement, little disease is seen in other organs. Observations were also made on the site of inoculation, as well as inguinal, tracheobronchial, and retrosternal lymph nodes. The

** Arlacel A – Product of Atlas Powder Co., Wilmington, Del.

FIG. 1. Photograph of formalin fixed spleens from 4 groups of animals taken from the experiment recorded in Table IV.

Group B—vaccinated with 0.05 mg of defatted bacilli
" C—alcohol-ether soluble fraction
" D—1.0 mg of lyophilized BCG
" E—non-vaccinated controls

Spleens are arranged in approximate order of decreasing amount of gross disease reading and numbering from left to right. Typical Feldman Index values for spleen are as follows. Group B—First 4 spleens, value 20; spleens 5, 6 and 7, value 15; spleens 8 through 13, value 10; spleens 14 through 17, value 5; and spleens 18 and 19, value 0. Group C—All spleens given value of 35 with exception of last 2 which were recorded as 30 and 25 respectively. Group D—Spleen 1, value 20; spleen 2 through 5, value 15; spleens 6 and 7, value 10; spleens 8 through 12, value 5; and spleens 13 through 20, value 0. Group E—Spleens 1 through 18 recorded as 35; spleen 19 recorded as 30 (this group was reduced for photographic purposes by leaving out 6 spleens between spleens 1 and 2).

extent of gross disease was recorded according to the scheme of Feldman, slightly modified. A value of 5-10 was recorded for lungs on which less than 12 surface lesions could be counted. For greater than 12 lesions, values of 15 and 20 were assigned and when the number of lesions was too large to count, values of 25 or 30 were given depending upon the over-all appearance. By referring to Fig. 1, one can see numerical assignments typical of those made for spleen involvement.

Results. The purpose of the first experiment was examination of 3 variables, namely, age of culture to be extracted, concentration of ethanol used in the first cell treatment, and use of alpha tocopherol acetate as an antioxidant, for their effects on immunizing potency of a crude chloroform extract. Ninety bottles of modified Long's medium were inoculated with culture 199RB. Sixty were harvested after 3 weeks incubation, and the remaining 30 after 12 weeks incubation. Half of the cultures at each period were treated with 50% ethanol and half with 95% ethanol. A like division of cell mass was made for subsequent extraction with chloroform containing tocopherol or with reagent grade chloroform. This series of variables ultimately gave rise to the following eight crude lipid extracts: 1) 3W50T—extract of 3 week (3W) culture treated with 50% ethanol (50) and extracted with chloroform containing tocopherol (T), 2) 12W95—extract of 12 week culture (12W) treated with 95% ethanol (95) and extracted with chloroform, 3) 3W50, 3W95, 3W95T, 12W50, 12W95T, 12W50T.

Several observations may be made from the results of this experiment recorded in

TABLE I. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed 6 Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG*	18	—	26‖
3W50T†	16	+	40
3W50	16	+	40
3W95T	16	—	58‖
3W95	19	—	51
12W50T	16	+	44
12W50	18	+	36
12W95T	18	—	47
12W95	19	—	32¶
Control	19	—	43

* Lyophilized vaccine (Tice 968-P), 0.5 mg given twice 3 wk apart.

† All lipid fractions suspended in mineral oil containing 1 part in 20 of aquaphor (1 mg of lipid in 0.1 cc of adjuvant) in two doses 3 wk apart.

‡ Tuberculin test, 0.0002 mg of PPD intracutaneously, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 800 viable units (plate count) given in 1 cc intraperitoneally. All groups were sacrificed 6 wk after infection and extent of gross disease recorded according to the scheme of Feldman.

Autopsy index:

	Spleen	Lung	Liver
Extensive	35	30	25
Moderate	20	20	20
Slight	10	10	10

(Moderate lung 12 or more discrete tubercles, slight 2-11 tubercles given value of 10, 1 tubercle value of 5.)

‖ Significant at the 1% level.
¶ " " " 5% " .

Table I. There appears to be little or no immunizing value in any extract of 3-week-old cultures while at least one extract of the 12-week culture appeared to give some protection. Extracts prepared from 50% ethanol treated cultures uniformly caused the development of a high level of tuberculin hypersensitivity†† whereas no hypersensitivity developed in animals vaccinated with extracts from 95% ethanol treated bacilli or BCG. One extract (3W95T) appeared to lower the resistance of guinea pigs to subsequent infection with tubercle bacilli.

Extracts evaluated in the second test were modifications of the three substances which showed some evidence of activity in the first experiment. Two extracts were precipitated at –20°C from methanol-chloroform yielding –20°C soluble fractions (–20S) and –20°C insoluble fractions (–20I). A third extract was chromatographed on a Magnesol-Celite column packed with 85 g of adsorbent. The column was charged with 2 g of the 12W50 extract and eluted with 1800 ml amounts of the following series of solvents: petroleum ether (PE), petroleum ether-benzene (PEB), benzene (B), benzene-ether (BE), methanol (M) and chloroform (C). The hold-back volume (volume of solvent retained by the packed column) for the column was approximately 300 ml and eluates were collected as complete hold-back volumes. Methanol eluates were divided into chloroform soluble and chloroform insoluble parts.

It was not possible to demonstrate resistance lowering activity with any preparation

TABLE II. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG*	12	+ —	8
12W95† (1 inj.)	17	—	27
12W95	14	—	25¶
" -20I	18	—	20¶
" -20S	14	—	33
3W95T	16	—	26
" -20S	17	—	35
" -20I	14	—	33
12W50	14	—	19‖
" PeB-2	17	—	29
" Pe (5-6)	8	—	36
" PeB-1, Pe-3	15	—	27
" BE-1	13	—	30
" BE-3	15	—	25¶
12W50M,CHCl,S	14	—	32
" CHCl,I	15	—	26
Controls	18	—	36

* Fresh vaccine (Phipps strain) 1 mg given one time only with the first injection.

† Lipid fractions suspended in Baylol-Arlacel, 0.1 mg given in 2 doses 3 wk apart.

‡ Tuberculin test, 0.0002 mg PPD intracutaneously, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 700 viable units of strain 199RB subcutaneously in the inguinal region. Sacrificed 6 wk after infection.

‖ Significant at the 1% level.
¶ " " " 5% " .

†† Selected animals from hypersensitive groups were removed before challenge and maintained in separate quarters as non-challenge controls. They were autopsied at the same time as other groups and the tissues were cultured and found to be negative with respect to acid-fast bacilli.

TABLE III. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG (1 mg)*	14	+	11
12W50 (.1 mg)	20	+	24
" (1 mg)	19	+	15
" (2 mg)*	17	+	16
" -5I	18	±	22
" -20I	20	+	21
" -20S	15	—	32
" -column	17	—	36
" -column + defatted bacilli†	17	+	9
Defatted bacilli†	15	+	12
Controls	19	—	37

* Given one time only at the first inoculation (BCG fresh Phipps strain).

† Defatted bacilli were suspended in adjuvant so that each 0.1 cc would contain 0.05 mg of cells (dry wt). Unless stated otherwise all lipid fractions were given in adjuvant (0.1 mg in 0.1 cc).

‡ Tuberculin test, 0.0002 mg of PPD, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 800 viable units of strain 199RB in 1 cc of saline. The cultures were filtered through Whatman #1 filter paper and 10 cc syringes were used once only. The last 1 cc volume in each syringe was discarded. Six wk after infection all groups were sacrificed and the extent of gross tuberculosis recorded according to the scheme of Feldman.

in this test. From the Feldman index figures it appears that a significant level of resistance was stimulated only in groups vaccinated with BCG and to a lesser extent with 12W50 and 12W95-20I. However, no fraction separated from 12W50 by column chromatography possessed the level of activity found in the parent fraction. It is interesting to note that fraction 12W50 Pe(5-6) was later identified by infrared spectroscopy as a contaminating oil inadvertently introduced in one of the extraction steps. From this experiment it is to be concluded that an active immunizing component was not separated from 12W50 by chromatography. Among possibilities which may account for this failure are the following: to produce an immunizing effect in the guinea pig, several components separated on the column would have to be combined, or activity of the fraction is destroyed on the chromatographic column, or finally that the active material is not eluted from the column.

In the experiment in Table III a new column was prepared and the 12W50 fraction applied in the same manner. The column was developed with the same series of solvents, however, this time all eluates were collected in one receiver and evaporated to dryness. The residue collected in this manner was tested as such and also after combining it with bacillary residue remaining after neutral solvent extraction (defatted bacilli). In addition, the 12W50 fraction was precipitated at 5°C and –20°C.

Comparing the results of this experiment with the previous one, it can be seen that approximately the same level of immunizing activity was obtained with 0.1 mg amounts of the 12W50 fraction, the chloroform soluble fraction of 50% ethanol treated bacilli. When the amount of fraction injected was increased to 1 mg in two injections or 2 mg in one injection, a higher degree of resistance to infection was produced. Fractions precipitated at 5°C and –20°C appeared to contain all the activity of the parent fraction, but no increase in activity was noted. The pool of eluates from chromatographic separation of 12W50 had completely lost all the original activity, however, when mixed with defatted bacillary residue, significant increase in potency of the preparation was noted. Defatted bacilli in adjuvant produced an increased resistance which compares favorably with some of the best preparations thus far evaluated. Finally it should be noted that the most significant protection was produced in animals that had been rendered hypersensitive to the extent that they gave positive tuberculin reactions with 0.0002 mg of PPD.

The last experiment is one in which further investigations of the defatted bacilli were made. Determinations were made of the effect of heat (121°C for 20 min.), extraction with borate buffer and the effect of further extraction of the residue with alcohol-ether containing 3% HCl.

From the results, Table IV, it would appear that extraction of neutral solvent insoluble residue with alcohol-ether containing 3% HCl destroys the remaining activity of this substance, since neither the soluble portion,

Anderson's(10) "firmly bound lipids," nor the residue after this extraction, possessed any ability to increase resistance of guinea pigs to tuberculosis. In addition it appears that 0.05 mg of defatted bacilli is the minimal effective amount and that autoclaving does not greatly affect the activity of the preparation. The fraction soluble in borate buffer at pH 9.1 had an intermediate activity which might have resulted from the presence of small numbers of intact cells.

Discussion. One of the major problems encountered in an examination of biological properties of fractions of tubercle bacilli, is removal of intact bacilli from extracts and fractions, Raffel(11), Myrivik(12). Results of an earlier report of this work (Smith *et al.*) (13), in which a smaller number of animals were used, are open to question because filtration was the only method employed to remove intact bacilli from extracts. In this report, the same considerations may apply to loss of activity of the 12W50 extract following chromatography.

Results obtained after vaccinating guinea pigs with small amounts of defatted bacilli in adjuvant show that significant increase in anti-tuberculous resistance has taken place. Guinea pigs vaccinated with whole dead bacilli are not protected to the same extent. These results would imply that the vaccine is improved by removing neutral solvent soluble substances (*i.e.* Cord Factor). In another experiment, not reported here, neutral solvent soluble material of a strain other than 199RB did not significantly alter resistance of guinea pigs when tested under similar conditions.

It has been reported by Anderson(10) that after extracting tubercle bacilli with neutral organic solvents it is possible to remove 10% more lipid by treating the bacilli with alcohol-ether containing 3% HCl. When these so-called "firmly bound lipids" were removed from previously extracted bacilli, neither the soluble portion nor the residue possessed any immunizing activity. Accordingly, it would appear that rupture of lipo-protein bonds which hold the firmly-bound lipids to bacillary protein renders the resulting materials inactive with respect to their resistance inducing properties. It is also interesting to note that at this same stage of fractionation, the property of inducing a state of delayed hypersensitivity was also destroyed.

The fact that purified protein or lipid fractions have the ability to stimulate increased resistance to tuberculosis in mice and guinea pigs is shown by the work of Weiss and Dubos(2), Kropp and Floyd(14), Choucroun (15), and Youmans(16). The present work neither confirms nor refutes these earlier observations.

With limitations on space and animal facilities, it has been necessary to carry out evaluations *in vivo* on a screening basis using minimal numbers of guinea pigs for any one vaccine and a challenge-sacrifice type of experiment. Estimations of the extent of disease at autopsy were made according to the method of Feldman. Statistical studies by analysis of variance technic,‡‡ indicates that, for groups of 14-20 animals, 10 points on the Feldman scale represents a difference which is significant at the 5% level. Since the work is still in a preliminary phase it was felt that only limited microscopic examination of tissues could be made. However, a definite correlation was shown to exist between gross and microscopic appearance of spleens and the extent of gross disease recorded for animals in several groups (BCG, 12W50 and Controls) of the experiment recorded in Table II.

Finally attention should be drawn to results obtained with BCG vaccines in the four experiments reported in this paper. Using approximately 1.0 mg of fresh vaccine (Phipps strain) or lyophilized vaccine (Tice strain), significant protection was afforded by BCG in each test. This is perhaps best shown in Fig. 1 where the spleens of four groups of the experiment recorded in Table IV are arranged in the approximate order of decreasing extent of gross disease reading left to right.

Summary. 1. Comparison has been made in guinea pigs of ability of BCG and various extracts and fractions of the tubercle bacillus to stimulate increased resistance to tuber-

‡‡ Courtesy of the Numerical Analysis Laboratory, University of Wisconsin.

TABLE IV. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction§	Mean Feldman index‖
Defatted bacilli .01 mg	20	+	36
Idem .05 mg	19	+	17
" 1.0 mg*	18	+	29
DFB heated to 121°C 20 min.	17	+	27
DFB extracted (soluble) in Alc-ether (3% HCl)	17	—	62
Residue of above	20	+ —	51
Whole cells heated at 121°C 20 min.	19	+	36
DFB extracted with borate buffer	17	+ (min)	34
BCG*	19	+	11
Controls	19	—	63

* Given one time only at time of first inoculation.
† BCG given one time only 6 wk before challenge. 1 mg of 949BLP. (Lyophilized vaccine, Tice laboratories.)
‡ All fractions given as 0.05 mg in adjuvant unless otherwise indicated.
§ Tuberculin tested with 0.0002 mg of PPD.
‖ All animals infected with 300 viable units of culture 199RB in 1 cc of diluent subcutaneously over the thorax. Undiluted culture was first filtered through Whatman #1 filter paper. To reduce possible variations in challenge inoculum, 10 cc syringes were used only once and the last 1 cc volume of suspension in each syringe was not used.

culosis. 2. Significant resistance was induced by 1 mg of BCG, given subcutaneously, in each of 4 experiments reported. 3. Comparable resistance was induced by 0.1 mg (in adjuvant) of a crude chloroform extract, and also by tubercle bacilli which had been exhaustively extracted with neutral organic solvents. 4. The immunizing potency of such defatted bacilli was completely destroyed by extraction with alcohol-ether containing 3% HCl and only partially by autoclaving for 20 minutes. The ability of defatted bacilli to induce delayed hypersensitivity was also destroyed by removal of firmly bound lipids with alcohol-ether containing HCl.

1. Bloch, H., and Noll, H., *Brit. J. Exp. Path.*, 1955, v36, 8.
2. Weiss, D., and Dubos, R., *J. Exp. Med.*, 1955, v101, 313.
3. Wong, S., and Weinzirl, J., *Am. Rev. Tuberc.*, 1936, v33, 577.
4. Randall, H. M., Smith, D. W., Colm, A. C., and Nungester, W. J., *ibid.*, 1951, v63, 372.
5. Randall, H. M., Smith, D. W., and Nungester, W. J., *ibid.*, 1952, v65, 477.
6. Smith, D. W., Harrell, W. K., and Randall, H. M., *ibid.*, 1954, v69, 505.
7. Kubica, G. P., Randall, H. M., Smith, D. W., in press.
8. Salk, J. E., and Laurent, A. M., *J. Exp. Med.*, 1952, 429.
9. Feldman, W. H., *Am. Rev. Tuberc.*, 1943, v48, 248.
10. Anderson, R. J., *The Harvey Lectures*, 1939, v35, 271.
11. Raffel, S., and Forney, J. E., *J. Exp. Med.*, 1948, v88, 485.
12. Myrivik, Q., and Weiser, R. S., *J. Immunol.*, 1952, v68, 413.
13. Smith, D. W., Grover, A. A., and Nungester, W. J., *Univ. Mich. Med. Bull.*, 1953, v19, 122.
14. Kropp, G. V., and Floyd, C., *Yale J. Biol. and Med.*, 1947, v20, 27.
15. Choucroun, N., *Am. Rev. Tuberc.*, 1947, v56, 203.
16. Youmans, G. P., National Tuberculosis Assn. Meeting, May 1955.

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Effect of Prolonged Oral Administration of Reserpine (Reserpoid®) on Gastric Secretion.* (22119)

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An increasing number of reports in medical literature testify to the effectiveness of *Rauwolfia serpentina* Bentham and its alkaloid, reserpine, in the treatment of hypertension, psychoses, neuroses and other conditions in which anxieties, tension and aggressions play a prominent role either in symptomatology or etiology. Reserpine might also be useful in the treatment of certain gastrointestinal disorders, such as peptic ulcer, functional bowel distress, including irritable colon syndrome with diarrhea or constipation, and other types of functional digestive disturbances. The work of Bein(1,2) indicates that reserpine acts upon the central autonomic mechanisms and that this action can be explained by a direct depressant effect on the hypothalamus. This produces peripheral effects similar to those resulting from stimulation of the parasympathetic nervous system. For example, Bein(3) and Plummer *et al.*(4) observed increased motor activity of intestinal tract in animals after administration of reserpine. More important, Plummer *et al.*(4) found that an increase in both volume and hydrochloric acid content of the gastric juice of dogs followed intravenous administration of 15 mg/kg of body weight of reserpine. This increased secretion was effectively prevented for several hours by parenteral use of an anticholinergic drug. Beman(5) stated that both oral and intravenous preparations of reserpine have a potent effect on parietal cells of gastric glands in man, as shown by a significant elevation of gastric acidity, not altered by administration of atropine.

Since it is recognized that free hydrochloric acid is one of the essential factors in production of peptic ulcer and that increased gastric acidity is more common in patients with duodenal ulcer than in normal subjects, it is of importance to determine whether prolonged oral administration of reserpine in therapeutic doses produces significant alterations in gastric acidity. It is the purpose of this paper to report effects of daily administration of reserpine orally on gastric secretion of 20 patients with various gastrointestinal disorders, including peptic ulcer.

Materials and methods. Twenty unselected patients from the Gastrointestinal Clinic, diagnosed as having duodenal ulcer, gastric ulcer, functional bowel distress, etc. (Table I), were given 0.25 mg reserpine† orally 4 times daily for 7 to 210 days; 8 were males and 12 females; age ranged 29 to 73 years. Patients with peptic ulcer were maintained on a modified Sippy diet with antacid medication. They received no anticholinergic drugs or other agents known to affect gastric secretion during this study. A one-hour basal gastric analysis was performed on each patient prior to therapy. The technic has been described previously(6). It consists first of collection via a Rehfuss tube of all gastric juice in the stomach after an over-night fast. This is called the residual specimen (R). Continuous aspiration is then carried out by means of hand syringe for 4 consecutive 15-minute periods. The (R) and each 15-minute collection (B1, B2, B3, B4) are titrated for free hydrochloric acid; total volume is measured and output of hydrochloric acid calculated. After the control basal gastric analyses were made, patients were given 0.25 mg of reserpine orally 4 times a day for variable lengths of time, and a second basal gastric analysis obtained. The results were compared with corresponding pre-treatment values.

Results. As shown in Table I, maximum free gastric acidity rose in 6 instances, fell in 13 instances, and was unchanged in one. Patients

* This study was supported by grant from The Upjohn Co., Kalamazoo, Mich.

† Supplied as Reserpoid® tablets by The Upjohn Co., Kalamazoo, Mich.

TABLE I. Individual Comparisons of Basal Gastric Analyses before and after Reserpine Therapy in 20 Patients.

Patient, sex, age and diagnosis		Before reserpine Res.	B1	B2	B3	B4	No. days on drug	During reserpine Res.	B1	B2	B3	B4
P.P. ♀ 72	HCl*	13	20	34	23	16	7	18	52	74	45	63
Duodenal ulcer	Vol.†	10	24	20	17	14		8	8	7	3	6
J.S. ♂ 73	HCl	0	19	38	30	8	9	0	20	20	8	0
Hiatus hernia	Vol.	32	13	11	3	8		4	15	14	5	8
K.B. ♀ 52	HCl	14	0	2	1	0	14	2	0	21	27	12
Gastric ulcer	Vol.	17	10	8	9	7		57	16	13	10	14
M.H. ♂ 70	HCl	4	42	44	44	36	14	19	40	20	26	24
Duodenal ulcer	Vol.	10	5	7	5	4		14	7	5	8	19
W.M. ♀ 56	HCl	60	43	70	85	85	14	34	75	72	72	63
FBD‡	Vol.	58	40	37	28	33		15	70	23	23	23
J.D. ♂ 54	HCl	1	0	7	10	7	17	0	0	0	0	0
Duodenal ulcer	Vol.	26	24	16	10	14		26	30	23	27	12
S.C. ♂ 40	HCl	18	21	40	10	4	24	12	18	26	38	28
FBD	Vol.	49	20	12	20	20		30	16	23	12	20
D.A. ♀ 62	HCl	0	26	17	17	16	28	0	30	28	30	14
FBD	Vol.	24	24	16	23	20		32	30	15	13	18
P.Mc. ♀ 71	HCl	0	0	0	0	4	31	0	0	3	3	0
FBD	Vol.	6	2	12	13	11		69	34	17	21	11
E.B. ♀ 54	HCl	0	9	19	48	48	31	0	0	6	16	10
FBD	Vol.	34	16	15	11	15		9	16	8	8	5
R.M. ♀ 63	HCl	0	35	50	70	45	35	0	2	37	56	34
FBD	Vol.	2	9	5	10	3		16	7	12	8	13
G.G. ♂ 60	HCl	25	20	25	35	40	37	0	0	0	0	12
Gastric resection	Vol.	4	10	20	22	20		5	18	11	13	13
P.E. ♂ 29	HCl	0	6	24	18	26	42	0	0	0	0	0
Gastric resection	Vol.	14	8	7	6	6		10	5	3	9	9
A.D. ♂ 57	HCl	0	41	65	47	84	42	48	67	69	29	70
FBD	Vol.	14	26	23	23	13		9	11	27	49	23
M.S. ♀ 43	HCl	8	0	0	0	0	46	10	0	18	4	15
Hiatus hernia	Vol.	24	38	16	15	18		24	30	32	21	22
C.G. ♂ 67	HCl	0	14	5	0	3	80	11	5	9	16	6
Duodenal ulcer	Vol.	10	6	6	6	4		10	8	7	7	3
M.A. ♀ 65	HCl	17	30	28	32	30	175	0	19	19	24	22
FBD	Vol.	20	25	32	18	43		6	17	10	19	16
M.O. ♀ 65	HCl	15	24	18	17	26	210	0	45	26	20	14
FBD	Vol.	27	15	17	30	25		14	15	12	5	5
M.Mc. ♀ 63	HCl	0	12	20	12	15	52	0	0	0	6	20
FBD	Vol.	33	22	17	8	5		21	43	28	25	22
O.W. ♀ 46	HCl	16	0	15	0	13	25	10	15	6	1	9
Pylorospasm	Vol.	40	10	28	12	47		42	17	15	15	12

* Conc. in mEq/L. † In ml. ‡ FBD = Functional bowel distress.

with peptic ulcer did not appear to react differently from those with other digestive disturbances or diseases. It is apparent that reserpine does not produce a consistent gastric secretory change in either direction. The average volume, free acidity and output of hydrochloric acid of residual specimens and of the four 15-minute basal specimens during reserpine administration showed no significant change from control values.

None of these patients experienced any significant side effects. An occasional patient complained of a slight "stuffy" feeling in the nose.

Discussion. Prior to clinical trial of reserpine in peptic ulcer and other gastrointestinal disorders, we investigated the effect on gastric acidity of prolonged oral administra-

tion of the drug in average therapeutic amounts. Although animal experiments, theoretical considerations, and one report on human volunteers have indicated that reserpine can increase gastric acidity, under conditions of this study, no significant change of volume, free hydrochloric acid or output of hydrochloric acid was observed during reserpine administration.

It is possible that large doses of reserpine taken orally or parenterally over a short period of time may increase gastric acidity, since they have been shown to cause other severe side effects, as indicated by Noce *et al.* (7). It is also possible, however, that tranquilizing effect of well-tolerated doses of reserpine may bring about a secondary decrease in gastric acidity and may therefore be helpful in the management of the peptic ulcer patient.

Conclusions. 1. Basal gastric analyses were made in 20 patients with various gastrointestinal disorders, including peptic ulcer, before and during therapy with reserpine. 2. No consistent change in volume, free hydrochloric acid concentration or output of hydrochloric acid was observed after prolonged oral administration of 0.25 mg of reserpine 4 times daily.

1. Bein, H. J., *Experientia,* 1953, v9, 107.
2. Bein, H. J., Gross, F., Tripod, J., and Meier, R., *Schweiz. med. Wchnschr.,* 1953, v83, 1953.
3. Bein, H. J., cited in Ref. 4.
4. Plummer, A. J., Schneider, J. A., Earl, A., Trapold, J., and Barrett, W., *Ann. New York Acad. Sc.,* 1954, v59, 8.
5. Beman, F. M., Kroll, H. C., and DeLor, C. J., *Clin. Res. Proc.,* 1955, v3, 131.
6. Rider, J. A., Gibbs, J. O., Ranson, W. A., and Swader, J. I., *J.A.M.A.,* 1955, v159, 1085.
7. Noce, R. H., Williams, D. B., and Rapaport, W., *ibid.,* 1955, v158, 11.

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Effects of Liver Fractions on Thyroid Function of the Rat.*† (22120)

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Several investigators have reported that diets containing liver produced thyroid enlargement in different laboratory animals (1-5). Hou(5) was able to demonstrate that goitrogenic activity was present in the ethanol soluble portion of ox liver. Although substantial thyroid enlargement was obtained in the latter investigation, a search of the literature has failed to yield any further information concerning the substance(s) responsible for the goitrogenicity. Anselmino and Hoffman(6) found fetal blood and other animal tissues to contain an "antithyroid protective" substance capable of overcoming the increase in basal metabolic rate caused by thyroxine injection. More recently Ershoff (7-10) has studied the "antithyrotoxic" effect of liver in overcoming the growth retardation of rats fed thyroidactive materials.

The present investigation is part of a study designed to identify the substance(s) responsible for the goitrogenicity of liver and to determine whether its goitrogenicity is related to the "antithyroid protective" substance or the "antithyrotoxic" factor.

Experimental. Sixty male and 60 female rats of the Wistar strain (housed in individual wire cages) were divided equally into 12 groups of 10 each and treated as indicated in Table I. Pork liver fractions‡ were incorporated into an iodine deficient ration (Table II) and into the same ration containing adequate iodine. The liver fractions replaced

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‡ Desiccated pork liver and the water soluble and water insoluble fractions of pork liver were purchased from Wilson Laboratories, Chicago, Ill.

TABLE I. Effect of Liver Feeding on Thyroid Function.

Treatment	Iodine content of ration, p.p.b.	Thyroid wt /100 g body wt, mg*	Thyroid-iodine content† Total, %	Thyroid-iodine content† Protein bound, %	Protein bound plasma iodine/100 ml, μg	Avg weekly gain, g
Basal ration	58	7.44 ± 1.21	.0106 ± .0025	.0103 ± .0025	1.40	29.2
3% H_2O soluble liver	"	8.11 ± 1.36	.0133 ± .0032	.0117 ± .0032	1.62	29.2
6% " " "	"	8.52 ± 1.08	.0094 ± .0025	.0087 ± .0018	1.14	26.8
20% H_2O insoluble liver	"	12.28 ± 1.42	.0040 ± .0004	.0029 ± .0008	1.20	28.6
20% desiccated liver	"	12.15 ± 2.15	.0038 ± .0028	.0025 ± .0014	1.62	14.6
.01% thiouracil	"	15.83 ± 2.59	.0022 ± .0009	.0013 ± .0007	1.21	27.3
Basal ration	400	5.77 ± 1.49	.0545 ± .0080	.0463 ± .0148	1.77	28.2
3% H_2O soluble liver	"	5.52 ± .76	.0616 ± .0115	.0579 ± .0120	2.45	29.8
6% " " "	"	6.44 ± 1.47	.0576 ± .0120	.0567 ± .0179	2.71	26.2
20% H_2O insoluble liver	"	6.58 ± .70	.0639 ± .0307	.0541 ± .0295	2.70	28.8
20% desiccated liver	"	6.39 ± 1.01	.0615 ± .0291	.0594 ± .0288	3.02	16.2
.01% thiouracil	"	10.39 ± 1.30	.0096 ± .0029	.0082 ± .0023	1.89	27.4

* Mean ± stand. dev., 10 animals/group.
† Mean ± stand. dev., from analyses of thyroids from 4 animals/group.

wheat germ in the diet and the percentage composition of the diets was adjusted by varying the quantity of corn and wheat germ until each contained the same percent protein. Diets containing 0.01% thiouracil were used to compare the goitrogenic activity of the liver fractions with that of a known goitrogen. After the animals had been on the experimental diets for 39 or 40 days they were starved for 20 hours after which basal metabolic rates were determined using a modified Haldane apparatus. On the 42nd day blood samples were taken and pooled according to treatment. Thyroid glands were excised, weighed and reserved for analysis. Protein bound plasma iodine determinations were made using the procedure of Barker (11). The following determinations were made on thyroid samples: total fresh weight, dry matter content, total iodine content, and protein bound iodine content.

TABLE II. Composition of the Basal Diet.*

Ingredient	%
Corn	58.6
Wheat germ†	35.0
Cottonseed oil	2.0
DL methionine	.4
Mineral mixture‡	4.0

* The following amounts of vitamins were added to this diet (expressed in mg/100 g): riboflavin 1, thiamine hydrochloride 0.75, niacin 5, *i* inositol 30, pyridoxine hydrochloride 1, choline chloride 200, biotin 0.01, folic acid 0.01, calcium pantothenate 5, *p*-amino benzoic acid 30, vit. B_{12} 0.002, 2-methylnaphthoquinone 0.50, α-tocopherol 10, vit. A acetate 0.05, and calciferol 0.005.

† Wheat germ which had been partially defatted, dehydrated and pulverized was purchased from Viobin Corp., Monticello, Ill.

‡ The mineral mixture had the following percentage composition: sodium chloride 4.43, magnesium sulfate (anhydrous) 6.78, disodium phosphate 8.86, monopotassium phosphate 19.03, potassium chloride 10.40, calcium phosphate monobasic 13.75, calcium lactate 33.07, ferrous ammonium sulfate 3.63, manganese sulfate 0.016, copper sulfate 0.024, and zinc carbonate 0.024.

Results. The goitrogenic activity of pork liver was found to be present in the water insoluble fraction and, with rats fed low iodine diets, was of nearly the same magnitude as that produced by 0.01% thiouracil. When the iodine intake was adequate the goitrogenicity of water insoluble liver was completely overcome. Desiccated liver exhibited the same degree of goitrogenicity as water insoluble liver and rats fed the former grew poorly. The concentration of iodine in the thyroid glands of rats fed the goitrogenic liver fractions and low dietary iodine was approximately one-third that of the corresponding controls. This decrease appeared to be due to lowered protein bound thyroid iodine. However, when adequate iodine was included in the diet the thyroid iodine of animals fed the goitrogenic liver fractions was the same as, or slightly higher than, that of the control animals fed the same level of iodine. Thiouracil reduced thyroid iodine concentration at both dietary iodine levels. Histological examination of thyroid sections indicated that

water insoluble liver was as active as 0.01 percent thiouracil in causing loss of colloid, and epithelial hypertrophy and hyperplasia. Slight variation was observed in protein bound plasma iodine values at the low dietary iodine level, but adequate dietary iodine resulted in increased plasma protein bound iodine values with rats fed all the liver fractions. This was not accompanied by an increase in basal metabolic rate. No significant differences in basal metabolic rate were observed between treatments when expressed either as a function of body weight or of body surface area.

Discussion. Rats fed low dietary iodine and water insoluble liver had greatly reduced protein bound thyroid iodine stores but when the liver fed animals received adequate dietary iodine not only did thyroid protein bound iodine return to normal but plasma protein bound iodine values were nearly doubled. This appears to indicate an increased synthesis and secretion of thyroxine rather than a decreased rate of thyroxine synthesis as is observed with thiouracil. The effects of the liver fractions on thyroid size and the level of protein bound iodine in the thyroid and in blood plasma seem to be dependent upon the level of iodine available to the thyroid. It would also appear that whatever causes this effect on thyroid and plasma protein bound iodine is either partially water soluble or different than the agent responsible for the thyroid enlargement observed in rats fed water insoluble or desiccated liver. In either case it appears that the mode of action of the goitrogenic factor(s) in liver is unlike that of thiouracil.

Summary. The present study is part of an investigation initiated in order further to purify and evaluate the goitrogenic substance (s) present in liver. The activity was found to be in the water insoluble portion of pork liver. Increased dietary iodine completely overcame its effect on thyroid size and iodine content. The decreased thyroid iodine of rats fed this material and inadequate iodine appears to be due to a fall in the protein bound fraction. This may be caused by an increased thyroxine secretion rate. Further experiments are in progress further to purify and study the metabolism of the active principle.

1. Burget, G. E., *Am. J. Physiol.*, 1917, v44, 492.
2. Hunt, R. J., *J. Am. Med. Assn.*, 1911, v57, 1032.
3. Marine, D., *J. Exp. Med.*, 1914, v19, 70.
4. Remington, R. E., *J. Nutrition*, 1937, v13, 223.
5. Hou, H. C., PROC. SOC. EXP. BIOL. AND MED., 1940, v43, 753.
6. Anselmino, K. J., and Hoffman, F., *Klin. Wochschr.*, 1933, v12, 99.
7. Ershoff, B. H., *Arch. Biochem.*, 1947, v15, 365.
8. ———, PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 209.
9. ———, *ibid.*, 1950, v73, 459.
10. ———, *ibid.*, 1950, v74, 391.
11. Barker, S. B., *J. Biol. Chem.*, 1948, v173, 715.

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Immunochemical Determination of Orosomucoid.* (22121)

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A low molecular weight, acidic glycoprotein having the mobility of an alpha-1-globulin at pH 8.6 has been isolated from human serum and has been extensively characterized(1-5). This protein, designated orosomucoid(5,6), is a major component of the "seromucoid"(7) or "mucoprotein"(8) fractions of serum, fractions which increase significantly in a number of diseases(6).

Studies on the concentration of orosomucoid in serum and other body fluids, as well as in various tissues, may be expected to yield information relating orosomucoid to disease and bearing on the origin of this glycoprotein. An immunochemical determination seemed best suited to the determination of orosomucoid in solutions containing proteins having similar stability and solubility characteristics. Previous attempts to prepare precipitating antibodies to this protein have been only moderately successful(9,10), due to the low antigenicity of orosomucoid. In the present paper it is shown that precipitating antibodies against orosomucoid can be prepared in chickens, and that these can be used to quantitate orosomucoid in serum and in tissue extracts.

Materials and methods. Orosomucoid was prepared from fraction VI of pooled normal human serum‡ by the method of Kefalides and Winzler(5). Antiserum was produced in healthy hens about one year old. One hundred mg of orosomucoid dissolved in 3 ml of water and mixed with an equal volume of aluminum hydroxide adjuvant was injected into the breast muscle. Two weeks later the injection was repeated, and 7 days later the chickens were bled out by cardiac puncture. The blood was citrated to prevent coagulation and to obtain maximum yields, and the cells separated by centrifugation. One hundred fifty mg of solid sodium chloride was added for each ml of plasma. The suspension was centrifuged and the fibrinogen which was salted out was removed as a gelatinous mat at the surface. The serum was then diluted with an equal volume of water to make the final sodium chloride concentration about 8%. The procedures developed by Goodman *et al.*(11-13) were adapted for the present immunochemical studies. Qualitative tests were carried out by adding a constant amount of antiserum (0.2 ml of a 1:2 dilution of antiserum) to 0.2 ml portions of serial dilutions of antigen in 8% NaCl, and examining for the presence of a precipitate after 1 hour at 38°C and again after 18 hours at 4°C. Quantitative determinations were carried out in 8% NaCl by adding 0.5 ml of the antiserum preparation to 0.5 ml of the antigen dilutions. The reactants were incubated at 38°C for 1 hour and then placed in a refrigerator for 18 hours. The tubes were then centrifuged at 2500 rpm for 30 minutes. Supernatants were decanted and saved for tests for antigen or antibody excess. Precipitates were resuspended in 5 ml of 8% saline and centrifuged again. A control containing antigen and normal chicken serum, as well as one containing 8% saline and the antiserum were carried out with each series of determinations. In no case did significant precipitation occur in the controls. The amount of protein in the precipitates was determined either from nitrogen determination by the method of Lanni *et al.*(14), using a factor of 6.25 to convert nitrogen values to

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TABLE I. Quantitative Orosomucoid-Antiorosomucoid Interactions.

Orosomucoid, μg	Total protein in precipitate, μg	Orosomucoid precipitated, μg	Antibody precipitated (by difference), μg	AB/AG	Supernatant tests, excess
0	0	—	—	—	AB
.7	13.8	.7	13.1	19.2	"
1.4	26.6	1.4	25.2	18.0	"
2.8	50.0	2.8	47.2	16.9	"
5.5	75.0	5.5	69.5	12.6	"
11	100.6	11.0	89.6	8.2	"
21	126.2	—	—	—	AG
42	158.2	—	—	—	"
84	113.4	—	—	—	"
167	94.2	—	—	—	"

0.5 ml antiserum PC-7 (diluted 1:2) plus 0.5 ml orosomucoid solutions in 8% NaCl. (Incubation 1 hr, 38°C, 18 hr 4°C. Precipitated nitrogen determined and converted to protein by multiplying by 6.25.)

protein, or by the method of Lowry *et al.* (15) using serum albumin as the standard. The serum seromucoid fraction was determined by the method of Weimer and Moshin(16).

Results. The antigenicity of orosomucoid is rather low in comparison with other plasma proteins. Preparation of antiserum from different chickens varied markedly, containing from 100 to 400 μg of antibody protein per ml.§ The preparation used to obtain the data shown in Table I contained between 90 and 100 μg of antiorosomucoid in 0.5 ml of the 1:2 dilution of the antiserum.

Table I shows a typical set of results when pure orosomucoid is mixed with antiserum prepared against it. As with other chicken precipitin systems(9) in which the reaction is carried out in concentrated NaCl, a relatively large excess of antigen is needed before inhibition of precipitation begins. The ratio of antibody to antigen in the precipitate formed in the antibody excess zone ranged from 19.2 to 8.2. Amounts of precipitate in this antibody excess zone, when plotted, serve as a calibration curve for determining the orosomucoid content of unknown mixtures. Since as little as 0.7 μg of the antigen yields a precipitate 19 times its own weight, it is unlikely that the precipitate results from a reaction with a minor component of the antigen.

To ascertain the homogeneity of the orosomucoid-antiorosomucoid system, the precipitation reaction was studied in a gel medium by the technic developed by Oudin(17). Two types of antisera were employed in these studies, antiorosomucoid and antibodies against fraction VI from which orosomucoid was prepared. This fraction contains albumin as well as several α-1 and α-2 proteins in addition to orosomucoid. The results of the studies using the Oudin technic are summarized in Table II. Since single bands were observed in series 1, 2 and 3, it appears that the orosomucoid-antiorosomucoid system is a homogeneous one. This band had a rate of migration which was proportional to the square root of the time, as shown by Munoz and Becker(18).

TABLE II. Orosomucoid-Antiorosomucoid Interactions in a Gel System.

In gel	Overlying solution	Results
1:2 dilution of antiserum in 8% NaCl, 0.15 M pH 7.4 phosphate & 0.6% agar	1% sol. of antigen in 8% NaCl	
1. Antiorosomucoid	Orosomucoid	Single strong band
2. "	Fraction VI	*Idem*
3. Antifraction VI	Orosomucoid	Single weak band
4. "	Fraction VI	Four distinct bands

§ It has been subsequently found that 4 to 8-fold higher antibody titers can be obtained by a single intramuscular injection of 200 mg of orosomucoid to chickens and collection of the blood in 10 days. It is likely that other injection schedules may result in still more effective antisera.

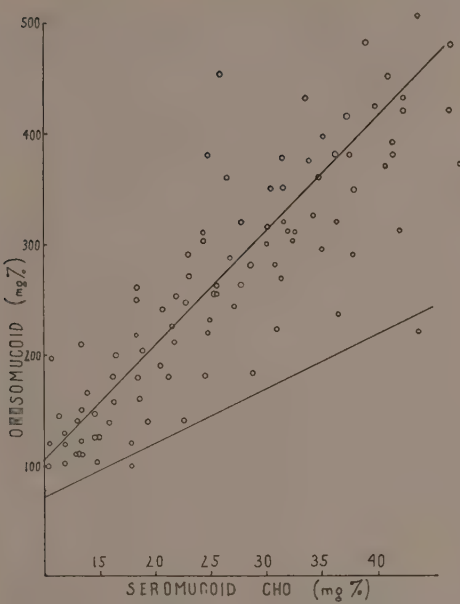

FIG. 1. Comparison of seromucoid fraction and orosomucoid levels in normal and abnormal sera.

The orosomucoid content of serum can be estimated by the immunochemical method described. Two-tenths ml of serum is diluted to 40 ml with 8% NaCl. Five-tenths ml of the diluted serum is added to 0.5 ml of 1:2 antiserum, and the incubation and protein determination carried out as previously described. The amount of orosomucoid is then determined from calibration curves of the type shown in Table I. Fifty normal sera examined in this manner showed a mean value and standard deviation of 103 ± 26 mg%. A single sample of serum was assayed repeatedly over the course of several months and gave consistent results. Thus, serum orosomucoid appears to be stable to storage by refrigeration.

In many clinical conditions including cancer, tuberculosis, etc., the orosomucoid levels are significantly elevated. Detailed data on serum orosomucoid levels in disease will be considered in subsequent reports. Values for orosomucoid are slightly higher than those for the seromucoid fraction determined in perchloric acid filtrates of serum(6). This is due primarily to the fact that there is considerable (30-50%) coprecipitation of the seromucoid fraction with the proteins precipitated by perchloric acid. Since it has been shown that orosomucoid is a major component of the seromucoid fraction(19), studies on seromucoid and orosomucoid levels of a series of 100 normal individuals and patients with various diseases associated with high serum glycoprotein levels were carried out. The results, shown in Fig. 1, demonstrate a rough correlation between the serum levels of orosomucoid and of the seromucoid fraction. The lower line in Fig. 1 is that expected from orosomucoid values corrected to a carbohydrate basis by multiplying by .16 (the fraction of hexose in orosomucoid)(3). Since the values of the seromucoid fraction are low by 30 to 50% due to coprecipitation with other proteins during perchloric acid precipitation(8), a further correction of the seromucoid values has been made by multiplying by 0.6. This second correction, which depends in part upon the total protein value, results in the upper line shown in Fig. 1. The fact that most of the experimental points lie along this line suggests that orosomucoid is the major protein in the seromucoid fraction. This substantiates the observation to be reported elsewhere(19) in which the seromucoid fraction has been shown by immunological methods to contain orosomucoid.

Much of the deviation of individual points from the best straight line is undoubtedly due to differences in coprecipitation or to combined experimental errors of the two methods. Points far to the left of this line, however, may be due to a cross reaction of the antibody with proteins precipitated by perchloric acid.

The present results demonstrate that an increase in the levels of orosomucoid in the serum of patients with tuberculosis, cancer, and a variety of other diseases can be determined with a specific immunological method.

The specificity and ease with which this determination can be carried out should make it a useful adjunct for the study of the source and significance of the serum glycoproteins. A possible extension of this work involves the coupling of fluorescein isocyanate with the antibodies to orosomucoid and the investiga-

tion of the deposition of the labeled antibodies in freeze-dried sections of various tissues suspected of being sources of orosomucoid.

Summary. A homogeneous glycoprotein, orosomucoid, isolated from normal human serum was found to be weakly antigenic in chickens. The antibody-antigen system appeared to consist of a single antigen and its specific antibody. A procedure for determining immunochemically the orosomucoid content of serum has been described. The results show a reasonably good correlation of orosomucoid with the levels of the seromucoid fraction (mucoprotein) of normal and pathological serum.

1. Schmid, K., *J. Am. Chem. Soc.*, 1950, v72, 2816.
2. ———, *ibid.*, 1953, v75, 60.
3. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.*, 1950, v185, 561.
4. Smith, E. L., Brown, D. M., Weimer, H. E., and Winzler, R. J., *ibid.*, 1950, v185, 569.
5. Kefalides, N. A., and Winzler, R. J., submitted for publication.
6. Winzler, R. J., *Methods of Biochemical Analysis*, Vol. 2, Interscience Press, 1955, 279.
7. Rimington, C., *Biochem. J.*, 1940, v34, 931.
8. Winzler, R. J., Devor, A. N., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.
9. Jager, B. V., *Fed. Proc.*, 1953, v12, 723.
10. Kornberg, L., and Lipari, R., *ibid.*, 1955, v14, 469.
11. Goodman, M., Wolfe, H. R., and Norton, S, *J. Immunol.*, 1951, v66, 225.
12. Goodman, M., and Wolfe, H. R., *ibid.*, 1952, v69, 423.
13. Goodman, M., Wolfe, H. R., and Goldberg, R, *ibid.*, 1954, v72, 440.
14. Lanni, F., Dillon, M. L., and Beard, J. W., PROC. EXP. BIOL. AND MED., 1950, v74, 4.
15. Lowry, O. H., Rosenbrough, N. J., Farr, A. L, and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.
16. Weimer, H. E., and Moshin, J. R., *Am. Rev. Tuberc.*, 1953, v68, 594.
17. Oudin, J., *Compt. Rend. Acad. d. Sc.*, 1946, v222, 115.
18. Munoz, J., and Becker, E. L., *J. Immunol.*, 1950, v65, 47.
19. Weimer, H. E., and Winzler, R. J., submitted for publication.

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Inhibition Studies with *Streptococcus faecalis*. Quinolines. (22122)

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Farber and his associates(1) observed that the anti-folics may be used to treat leukemia. This observation gave impetus to the synthesis and examination of metabolite blocking compounds. One outgrowth of this was the observation by Hitchings and his co-workers (2,3) that some anti-folics may have antimalarial properties. In view of these findings we have examined compounds initially prepared for testing against malaria(4-9) for their ability to interfere with the utilization of folic acid.

In this communication the results of tests on 93 quinolines for anti-folic properties are given. It may be indicated at the outset that some of the most useful anti-malarials are without effect against folic acid. While some specificity of activity was suggested by the study, the compounds do not compare favorably with some of the known anti-folics. Accordingly, the results appear to be only of theoretical interest at this time.

Experimental. The method of test was based on the use of *Streptococcus faecalis* (ATCC No. 8043) with a slightly modified version of the medium of Teply and Elvehjem (10). The modifications consisted of omitting the xanthine from the medium and of substituting "vitamin-free" casein hydrolysate (Nutritional Biochemicals) for the charcoal treated preparation suggested by the above authors. Under our conditions of test, with increasing amounts of folic acid in the medium up to 1 mμg per ml of medium, the

TABLE I. Quinolines Which Interfere with the Utilization of Folic Acid.

Compound*	Derivatives	Activity vs. folic acid, μg/ml
4-$NH(CH_2)_2N(C_2H_{13})_2$	3-CH_3 7-Cl(4)	1.6/ 8
4-$NH(CH_2)_4N(C_2H_5)_2$	3-NO_2 7-Cl	40 /200
4-$CH(C_6H_5)COO(CH_2)_2N(C_2H_5)_2$(8)		"
4-$COO(CH_2)_2S(CH_2)_2N(C_2H_5)_2$	2-OC_4H_9	"
4-$COO(CH_2)_2S(CH_2)_3NC_5H_{10}$	"	"
4-$SCH_2CONH(CH_2)_2N(C_2H_5)_2$	3-CH_3 7-Cl(9), 3-Br 7-Cl(9), 3-CH_3 6-OCH_3(9), 3-CH_3 6-Br(9)	"
8-$NH(CH_2)_3N(C_2H_9)_2$	6-OCH_3	8 / 40

* Six publications, considered representative, are cited for the chemistry of compounds given in the Tables. A more complete listing of compounds tested against malaria may be seen in Wiselogle(12) or in the F. S. A. monograph(13).

growth, as judged by acid production, increased from blanks of about 0.5 ml of N/10 alkali per tube up to 9.5 to 10 ml of alkali. For the test, about half maximum growth was obtained with 0.32 mμg of folic acid per ml of medium. Accordingly, the test compounds were run against this amount of folic acid in the medium. They were added to duplicate tubes in amounts of 1.6, 8.0, 40 or 200 μg per ml of medium. The tubes were plugged, sterilized for about 12 minutes at 120°C, cooled, inoculated(11) with a drop of a suspension of the organism, and incubated for 3 days at 37°C. At the end of this time, the growth was estimated titrimetrically.

Results. The compounds tested and their ability to interfere with the utilization of folic acid are summarized in Tables I and II. An arbitrary ratio term is given in the Table to evaluate the compounds which interfere with the growth of the organism. The lesser amount is that at which little or no interference with the growth of *S. faecalis* occurred, while the larger amount is that at which growth was essentially completely prevented.

From the results summarized in Table I it may be seen that 10 of the quinolines were toxic to the organism. To determine the nature of the toxicity, the inhibitory activity was further differentiated. With 2.0 mμg of citrovorum factor(14) per ml of medium as the limiting nutrient, in place of folic acid, the mercapto quinoline N-(2 diethylaminoethyl) - (7-chloro-3-methylquinolyl-4) - mercaptoacetamide was no longer inhibitory. With 2.5 μg of thymine(15,16) per ml of medium as the limiting nutrient in place of folic acid 7 - chloro-4-(4-diethylaminobutylamino)-3-nitroquinoline was no longer inhibitory. Finally, with 2.5 μg of thymidine(17) per ml of medium as the limiting nutrient in place of folic acid, the mercaptoquinoline N-(2 - diethylaminoethyl) - (6 - methoxy-3-methylquinolyl - 4) - mercaptoacetamide was no longer inhibitory.

The inhibitory properties of the foregoing 3 quinolines, while interesting, are considerably below those of some of the better known anti-folics. For example, in the presence of 0.32 mμg of folic acid per ml of medium under the above conditions of test the inhibition ratios were 0.008/0.04 μg for aminopterin, 0.00032/0.0016 μg for amethopterin, and 0.0016/0.008 μg for pyrimethamine (Daraprim) per ml of medium. Thus the latter were 5000-fold (or more) as active as the above 3 quinolines. Accordingly, the results appear to be of no more than theoretical interest at this time.

Seven of the quinolines of Table I which blocked the utilization of folic acid were still inhibitory with thymidine in the medium as the limiting nutrient. Whether the inhibitory activity of these compounds was due to a block in the nucleic acid metabolic sequence or whether it was due to interference in some other metabolic sequence is not known.

The anti-malarials given in Table II, in amounts up to 200 μg per ml of medium, did not inhibit the organism in the presence of limiting amounts of folic acid. This included chloroquine (Aralen), hydroxychloroquine†

† Not to be confused with oxychloroquine.

(Plaquenil), sontoquine, primaquine, and isopentaquine. The biguanide anti-malarial Paludrine in our hands was likewise inert, although it is clearly an anti-folic with the *L. casei* test system(18). Thus, the foregoing anti-malarials differ from pyrimethamine, which is a strong inhibitor of folic acid with *S. faecalis*.

Accordingly, it may be concluded that an examination of 93 quinolines, prepared in

TABLE II. Quinolines Which Do Not Interfere with the Utilization of Folic Acid.

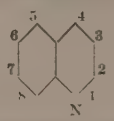
Compound 	Derivatives
a. 4-amino quinolines	
bis[(4-NH_2,2-CH_3,6-quinolyl)-NHCO] = C(/CH_3 \ C_3H_7)	
4-$NH(CH_2)_2NHCH_2CH_2OH$	7-Cl, 3-CH_3, 7-Cl
" $N(C_2H_5)_2$	3-CH_3 7-Cl
" $N(C_4H_9)_2$	"
4-$NH(CH_2)_3N(C_2H_5)_2$	7-Cl(5), 3-CH_3 7-Cl (brachysan)
4-$NHCH_2CHOH\ CH_2N(C_2H_5)_2$	7-Cl (oxychloroquin) (6), 3-CH_3
	7-Cl(6), 3-Br 7-Cl, 3-I 7-Cl, 5-Cl
4-$NHCH(CH_3)(CH_2)_2NC_4H_8O$	7-Cl
4-$NH(CH_2)_4N(C_2H_5)_2$	7-Cl(5), 7-Br(5), 6-OCH_3, 3-CH_3
	7-Cl (norsontoquine) (6)
4-$NH(CH_2)_4N(C_4H_9)_2$	3-CH_3 7-Cl
" $_2CHOHCH_2N(C_2H_5)_2$	7-Cl
4-$NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	" (chloroquine) (5), 7-Br(5),
	3-CH_3 7-Cl (sontoquine) (6), 3-CH_3
	7-Br(6), 2-CH_3 7-Cl, 5-Cl
" $N(C_2H_5)(C_2H_4OH)$	7-Cl (hydroxychloroquine)
" NC_4H_8	7-Cl
4-$NHCH_2CH(C_6H_4OCH_3)(CH_2)_2N(C_2H_5)_2$	3-CH_3 7-Cl
" $(C_6H_5)(CH_2)_2N(C_2H_5)_2$	"
4-$NH(CH_2)_2S(CH_2)_2N(C_2H_5)_2$	7-Cl(7)
" $SO_2(CH_2)_2N(C_2H_5)_2$	"
b. Carbon linked at position 4	
4-$CH(C_6H_5)CONH(CH_2)_2N(C_2H_5)_2$	7-Cl(8)
" $(CH_2)_2N(CH_3)_2$	5-Cl(8)
" $(CH_2)_2N(C_2H_5)_2$	7-Cl(8)
4-$C(C_6H_5)(CN)(CH_2)_3N(CH_3)_2$(8)	" , 6-OCH_3(8)
" $(CONH_2)(CH_2)_2N(CH_3)_2$	5-Cl(8)
" $(CONH_2)(CH_2)_2N(C_2H_5)$	7-Cl(8)
4-$CH(C_6H_5)COO(CH_2)_2N(C_2H_5)_2$	"
c. 4-mercaptoquinolines	
4-$SCH_2CONH(CH_2)_2N(C_2H_5)_2$(9)	7-Cl(9), 2-CH_3 7-Cl(9), 5-Cl(9),
	3-CH_3(9), 3,8-$(CH_3)_2$(9), 3-CH_3
	8-OC_2H_5(9), 6-OCH_3(9)
4-$S(CH_2)_3N(C_2H_5)_2$	7-Cl(4), 3-CH_3 7-Cl(4)
" NC_5H_{10}	7-Cl(4)
4-$SCH_2COO(CH_2)_2N(CH_3)_2$	7-Cl(9), 5-Cl(9)
" $N(C_2H_5)_2$(9)	" " , 6-OCH_3(9)
" NC_5H_{10}	" "
" NC_4H_8O	5-Cl(9)
4-$SCH(CH_3)COO(CH_2)_2N(C_2H_5)_2$	7-Cl(9)
4-$S(CH_2)_5NHC_3H_7$	" (4)
4-$S(CH_2)COO(CH_2)_2N(C_2H_5)_2$	" (9)
4-$S(CH_2)_2COO(CH_2)_3N(C_2H_5)_2$	" (9)
d. Oxygen linked at position 4	
4-OH	3-CH_3(6), 7-Cl 2-COOH(5)
4-$OCH(CH_3)(CH_2)_3N(C_2H_5)_2$	7-Cl(4)
4-$O(CH_2)_2S(CH_2)_2N(C_2H_5)_2$	7-Cl

TABLE II (continued).

Compound	Derivatives
e. 8-amino quinolines	
8-NH_2	6-OCH_3(amichin)
8-$NHCH(CH_3)CH_2NHC_3H_7$	6-OCH_3
8-$NH(CH_2)_3N(C_4H_9)_2$	"
" $(CH_2)_4NH_2$	"
8-$NHCH(CH_3)(CH_2)_3NH_2$	" (primaquine), 4-CH_3 6-OCH_3
" NHC_3H_7	" (isopentaquine)
" $N(C_2H_5)_2$	6-OH
8-$NH(CH_2)_5NH_2$	6-OCH_3
" $(CH_2)_2S(CH_2)_2N(C_2H_5)_2$	" (7)
" $(CH_2)_3S(CH_2)_2N(C_2H_5)_2$	" (7)
" $S(CH_2)_3N(C_2H_5)_2$	"
f. 2-mercapto quinolines	
2-$S(CH_2)_2N(C_2H_5)_2$(4)	
" $(CH_2)_3N(C_2H_5)_2$	4-CH_3(4)
g. Carbon linked at position 1	
1-$CH_2C_6H_4SO_2NH_2$	1-Br

connection with an anti-malarial program, has not disclosed compounds with immediate application.

Summary. 1. Ninety-three quinolines were tested against *Streptococcus faecalis* in the presence of limiting amounts of folic acid. Ten of the quinolines were toxic to the organism when they were in the medium at concentrations of 8.0 to 200 μg/ml. The toxicity of 3 of the 10 inhibitors could be abolished. The inhibition of N-(2-diethylaminoethyl)-(7-chloro-3-methylquinolyl-4) - mercaptoacetamide was reversed by citrovorum factor, that of 7-chloro-4-(4-diethylaminobutylamino)-3-nitroquinoline was abolished by thymine, and that of N-2-diethylaminoethyl-6-methoxy-3-methylquinolyl - 4) - mercaptoacetamide was overcome by thymidine. The remaining 7 were not studied further since their degree of inhibition did not compare sufficiently favorably with that of some of the better known anti-folics. 2. None of 5 anti-malarials examined (chloroquine, hydroxychloroquine, sontoquine, primaquine, and isopentaquine) interfered with the utilization of folic acid. Thus, these differ metabolically from pyrimethamine which has been shown to be both an anti-folic and an anti-malarial.

1. Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A., *N. Engl. J. Med*, 1948, v238, 787.

2. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., Sherwood, M. B., and VanderWerff, H., *J. Biol. Chem.*, 1950, v183, 1.

3. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., and VanderWerff, H., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1318.

4. Clinton, R. O., and Suter, C. M., *J. Am. Chem. Soc.*, 1948, v70, 491.

5. Surrey, A. R., and Hammer, H. F., *ibid.*, 1946, v68, 113.

6. Steck, E. A., Hallock, L. L., and Holland, A. J., *ibid.*, 1946, v68, 380.

7. Huber, W., Bair, R. K., Laskowski, S. C., Jackman, M., and Clinton, R. O., *ibid.*, 1946, v68, 322.

8. Cutler, R. A., Surrey, A. R., and Cloke, J. B., *ibid.*, 1949, v71, 3375.

9. Surrey, A. R., *ibid.*, 1948, v70, 2190.

10. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, v157, 303.

11. Black, T. L., and Arnold, A., *Ind. Eng. Chem., Anal. Ed.*, 1940, v12, 344.

12. Wiselogle, F. Y., *A Survey of Antimalarial Drugs*, Edwards, Ann Arbor, Mich., 1946.

13. Federal Security Agency, *Survey of Antimalarial Agents*, Public Health Monograph No. 9, 1953.

14. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

15. Stokes, J. L., *J. Bact.*, 1944, v48, 201.

16. Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.*, 1947, v170, 323.

17. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, 1948, v70, 2299.

18. Falco, E. A., Hitchings, G. H., Russell, P. B., and VanderWerff, H., *Nature*, 1949, v164, 107.

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Development of Spontaneous Hepatoma in Mice in Relation to Composition of Dietary Fat.*† (22123)

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The development of spontaneous hepatomas in C_3H mice has been shown to be enhanced by an increased amount of fat in the diet(7,10). Very little, however, is known about the dependence of this tumor on the nature of the dietary fat, of the type demonstrated for the azodye induced hepatoma of the rat(8). In the present study an attempt has been made to find if groups of fat components, derived from the same source, but separated from each other by physical means and fed in equivalent amounts would exert a specific action on the development of the mouse hepatoma.

Methods. Among several methods of fat fractionation(1-5) fractional crystallization from solvents(2,4) presents decisive advantages for nutritional studies. It does not subject fat to chemical reactions, the exact outcome of which it is impossible to predict without precise knowledge of composition of the original fat. It does not involve addition of more than one chemical, which if properly chosen as to its boiling point can be quantitatively removed. Nor does it require heating the fat to higher temperatures, as *e.g.*, molecular distillation. The procedure adopted was as follows: One pound of hydrogenated vegetable fat (H.V.F.)‡ was melted at 50°C, then dissolved in 2 lb of diethyl ether (Purified ether reagent, Merck, glass containers). Ether was chosen as solvent, rather than acetone, because of its lower boiling point. The resulting mixture had a temperature of 35°C. The mixture was cooled for 30-40 minutes under constant mechanical stirring until a temperature of +15°C was reached. The precipitate formed was filtered by gravity at that temperature (Fraction I), filtration requiring about 20 minutes. E. & D. filter paper No. 615, size 33 was used. Precipitate was not washed, but the mother liquor was pressed out as far as possible. The filtrate was cooled further and precipitate formed at +5 (Fraction II), —5 (Fraction III) and –15° (Fraction IV) filtered off similarly. Cooling times required were approximately 40, 50 and 180 minutes, respectively. Filtration time was approximately the same for all fractions. The precipitate obtained at the last temperature constituted "Fraction V." The residues were heated at low pressure until liquefied and remaining ether removed by vacuum distillation at the temperature of liquefaction. This required approximately 4 hours. The filtrate at –15°C was similarly freed of ether at 35°-40°C. Crude fractions thus obtained were allowed to solidify and kept refrigerated until their incorporation into diets. Titer ranges were determined on aliquots of each fraction and are given in Table I. Total yields for each fraction were determined on each lot processed and were found to vary little from one processing to another. Average values are also given in Table I. No attempt has been made to determine exact composition of individual fractions. However, on the basis of extensive analyses of fractions obtained by similar methods reported by other workers it is believed that fractions precipitating at higher temperatures contained a higher proportion of glycerides of long-chain, branched-chain

TABLE I. Melting Ranges and Approximate Recoveries of Hydrogenated Vegetable Fat Fractions.

Fraction	Melting range (°C)	Recovery* (g)
I	55-57	1480
II	44-49	1060
III	37-41	1120
IV	30-33	1040
V	12-26	2000

* Initial amount of material approx. 15000 g.

* Abstract in *Proc. of Am. Assn. of Cancer Research*, 1954, v1, 22.

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‡ H.V.F. = commercial Crisco.

TABLE II. Incidence of Hepatomas.

	H.V.F.	Lard	Fr. I	Fr. II	Fr. III	Fr. IV	Fr. V	P*
No. of tumor bearing mice	8	9	3	0	5	9	13	<.001
No. of mice bearing multiple tumors	1	3	1	0	1	1	3	
Total No. of tumors	9	12	4	0	6	12	20	
Size: <10 mm	4	6	0	0	2	8	12	
>10 mm	5	6	4	0	4	4	8	

* By the χ^2 method.
H.V.F. = Hydrogenated vegetable fat. Fr. = Fraction.

and saturated fatty acids and a smaller proportion of unsaponifiable matter. Composition of diet into which fractions were incorporated was as follows: casein 29%, glucose 34%, yeast 8.2%, Jones Foster salt mixture 4.1%, alfalfa leaf 4.1%, cod liver oil 2.1%, wheat germ oil 1%, neutral fat (lard, hydrogenated vegetable fat or one of the 5 fractions) 17.5%. The diet was made in batches sufficient to last for 3 months at a time. Fractionation was carried out each time a new batch of diet was required. Seven groups of 20 male C_3H mice each were used. Five were fed fractions of hydrogenated vegetable fat while 2 control groups received unfractionated and untreated H.V.F. and lard, respectively. Animals were started on experimental diets at weaning (3 weeks of age, average weight about 10 g). Members of the same litter were distributed among as many groups as possible. Animals were kept 2 to a cage and weighed at weekly intervals. They were fed *ad libitum* from a common food cup, which was left in the cage for one week at a time. At all times they had access to water. All cages were placed in the same part of the room and room temperature did not vary more than 8°F from day to day.

Results. The experiment was terminated after 54 weeks and autopsies performed on all animals. Table II gives the cumulative incidence of hepatomas up to that time. It also summarizes the occurrence of multiple tumors and the breakdown according to size. It can be seen that fractions of low melting point show a markedly greater incidence of hepatomas. Compared with fractions of high melting point there appears to be little corre-

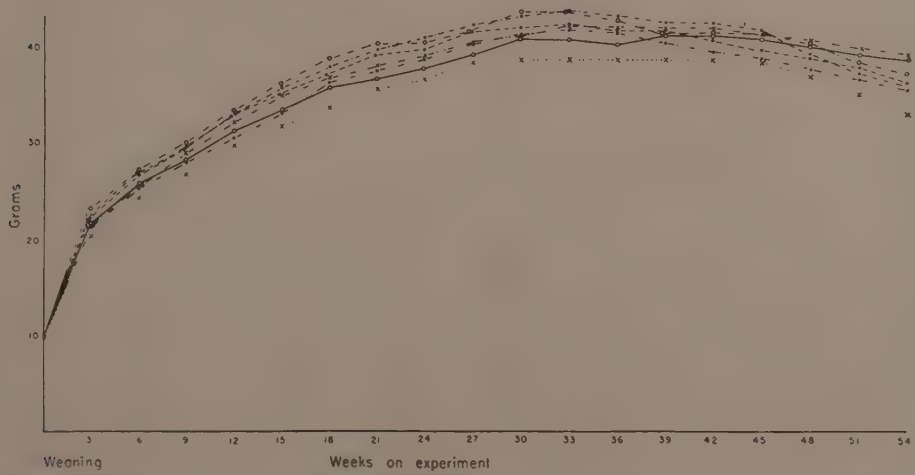

FIG. 1. Weight curves of mice fed different fractions of hydrogenated vegetable fat (H.V.F.). ——— lard, —••••— H.V.F., •••• Fraction I, —•••— Fraction II, —•— Fraction III, ——— Fraction IV, —••— Fraction V.

lation between tumor incidence and tumor size. The occurrence of multiple tumors is sufficiently rare in all groups so as not to permit any conclusions as to the presence of differences.

The foregoing data strongly suggest that the nature of the dietary fat may have a significant bearing on the development of the C_3H mouse hepatoma. The effect observed here is similar to the one reported by Shear and Leiter(9), who fractionated lard by filtration at 38°C and found that the liquid fraction when used as a carrier for benzpyrene gave rise to more tumors than the solid fraction.

It still remains to be established however, whether the differences in the effects are due to qualitative peculiarities of the different fractions or to differences in the actual amounts of fat absorbed. No simple correlation between weight and tumor incidence is apparent from a comparison of the weight curves given in Fig. 1 and the data in Table II. This would suggest a qualitative, rather than a quantitative, difference, but the question as to the nature of the responsible factor remains open for the present.

Summary. Five fractions of hydrogenated vegetable fat (commercial crisco) have been prepared by crystallization from ether at +15, +5, —5, and —15°C. When incorporated in the standard diet at equivalent levels, fractions of low melting point, presumably containing largely unsaturated and shorter-chain fatty acids as well as unsaponifiable matter, are found to give rise to a high proportion of hepatomas in C_3H male mice, while fractions of high melting point give rise to but a few. The magnitude of the observed difference in incidence ($p<.001$) is believed sufficient to justify further studies on the nature of the responsible factors.

1. Bailey, A. E., *Industrial oil and fat products*, pp. 70-75. New York: Interscience Publishers, Inc., 1945.

2. Bömer, A., Schemm, A., and Heimsoth, G., *Z. Unters. Nahr u. Genussm.*, 1907, v14, 90.

3. Henderson, J. L., and Jack, E. L., *Oil and Soap*, 1944, v21, 90.

4. Hilditch, T. P., and Maddison, L., *J. Soc. Chem. Ind.*, 1940, v59, 162.

5. ———, *ibid.*, 1951, v60, 258.

6. Jack, E. L., Henderson, J. L., Reid, D. F., and Lepkovsky, S., *J. Nutr.*, 1945, v30, 175.

7. Lee, Y. C. P., Ph.D. Thesis, Univ. of Minn., 1953.

8. Miller, J. A., *Ann. N. Y. Acad. Sci.*, 1947, v49, 19.

9. Schear, M. J., and Leiter, J., *Proc. Am. Soc. Biol. Chem.*, 1941, v35, 115.

10. Silverstone, H., and Tannenbaum, A., *Cancer Research*, 1951, v11, 200.

11. Tannenbaum, A., and Silverstone, H., In Greenstein, J. P., and Haddow, A., *Advances in cancer research*, pp. 452-497, New York, Academic Press, 1953.

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Collagenase Activity of Dog Pancreatic Juice. (22124)

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Pancreatic juice obtained from pancreatic fistulae artificially created in dogs produces striking digestion of collagen. This is not due to trypsin, chymotrypsin, or carboxypeptidase. It is the purpose of the present work to describe these studies.

Methods and materials. Adult mongrel dogs of both sexes were used. Under aseptic technic the abdomen of the anesthetized animal was opened through a midline incision and the pancreas exposed. The accessory pancreatic duct was dissected free, ligated, and cut, following which the main duct was identified. After placing a ligature close to the point of its entrance into the duodenum, the duct was cannulated with a polyethylene tube whose lumen measured 1.14 mm in diameter; this was tied securely in place. The tube was then brought out through a separate stab incision, following which a Witzel jejun-

ostomy was performed using a number 8 French rubber catheter which was brought through another stab incision. The wound was then closed in layers and a sterile condom was tied to the polyethylene tube. The jejunostomy tube was then securely clamped and a dressing applied. Following recovery from the anesthetic, secretions started flowing in 12-24 hours from the polyethylene tube reaching a maximum of 20 ml per hour in 36-48 hours. The animals could be kept alive for prolonged periods if the pancreatic secretions were returned through the jejunostomy tube. Otherwise they became dehydrated, anorexic and lethargic and died within 7 to 8 days. The secretions tested were collected within the first 24-48 hours in each instance and were also tested for sterility by routine bacteriologic methods. These were free of bacterial growth. Collagen was prepared by the method of Buechler(1) using fresh beef achilles tendon instead of steer hide. 100 mg of prepared tendon was suspended in 2.0 ml of secretion from a pancreatic fistula and incubated at 37°C for 5 hours. The suspension was then filtered and the precipitate washed. The precipitate was then dissolved in 9.0 ml of 0.5 N HCl and autoclaved for 30 minutes at 15 lb pressure. The solution was then made up to 10.0 ml volume. Collagenase activity was measured by the quantitative assay of the hydroxyproline in the residue as described by Neuman and Logan(2). Since hydroxyproline forms a constant fraction of collagen, this method is an accurate assessment of the amount of undigested collagen, permitting ready calculation of the amount of collagen digested. Commercially available preparations* of trypsin, chymotrypsin, and carboxypeptidase were used. 0.1 M phosphate buffers at pH 7.0 and 8.0 were used. 0.5 mg of each of the commercial enzymes were dissolved in 2.0 ml of buffer solution for testing the activity. Changes in collagen apparently occur at pH 4.5 and below and will permit digestion by trypsin and vegetable cathepsins(3). These findings have been confirmed by us and are not included in this report.

TABLE I. Percent Digestion of Collagen and Casein by Pancreatic Juice and Trypsin.

		% collagen digested	% casein digested
Pancreatic juice	Dog 1	52	0
	2	41.5	0
	3	45	0
Trypsin before heating	No. 1	16	96
	2	13	92
Trypsin after heating	No. 1	0	85
	2	0	79

Results. Unbuffered pancreatic juice was used in the tests on collagen. The usual pH range was 8.2-8.6. The digestion of collagen by dog pancreatic juice varied. Dog No. 1 digested 52.0%, No. 2 digested 41.5%, and dog No. 3, 45.0% (Table I). Commercially prepared trypsin, both at pH 7 and 8 had slight collagen digesting ability. Preparation No. 1 digested 16.0% and preparation No. 2 digested 13.0%. As pointed out by Northrop(4) and confirmed by us, heating to 95°C for 4½ minutes, then cooling to 20°C has little effect on the proteolytic activity of trypsin, as determined by the method of Grob(5). However, such heating markedly alters its ability to digest collagen. Neither trypsin preparation digested any collagen after such heating (Table I). When pancreatic juice was heated in the same fashion all collagenase activity disappeared. The inactivated pancreatic juice had no tryptic activity. Bacterial collagenase prepared from *Cl. histolyticum* was heated to 95°C for 4½ minutes then cooled, with complete loss of collagenase activity. Chymotrypsin and carboxypeptidase did not digest collagen at pH 7.0 or 8.0. Duodenal content, extract of duodenal mucosa and bile had little effect on the collagenase activity of the pancreatic secretion. The activating substance, if any, for collagenase activity remains unidentified.

Discussion. The marked collagenase activity of crude pancreatic juice was present in each of the dogs tested. This cannot be accounted for by the action of any of the pre-

* Commercial preparations used: Trypsin preparation no. 1: Tryptar, Armour Lab.; trypsin preparation no. 2: Trypsin, Worthington Biochemical Co., 2x crystallized Chymotrypsin, Worthington Biochemical Co.; Carboxypeptidase, 3x crystallized.

viously described proteolytic enzymes of the pancreas for 3 reasons: 1. The degree of digestion is at least 3 times as great as that obtained from trypsin. 2. Heating trypsin to destroy its collagen digesting ability, but not its proteolytic properties, reduces the amount of collagen digested to zero. 3. Pancreatic juice from a dog fistula which has not been activated by intestinal juice has no tryptic activity, nevertheless, it has marked collagenase activity. The inability of trypsin to digest any significant amount of collagen has also been reported by Neuman and Tytell(6).

These findings suggest that collagenase must be considered in the pathogenesis of pancreatitis and other pancreatic diseases.

Summary. Evidence has been presented to show that pancreatic juice of the dog has the ability to digest collagen. This ability is not due to the proteolytic enzymes of the pancreas, trypsin, chymotrypsin, or carboxypeptidase.

1. Jacobs, J. L., *J. Am. Leather Chem. Assn.*, 1949, v44, 722.

2. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.

3. Sherry, S., Troll, W., and Rosenblum, E. D., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 125.

4. Northrop, J. H., *J. Gen. Physiol.*, 1932, v16, 323.

5. Grob, D., *ibid.*, 1946, v29, 219.

6. Neuman, R. E., and Tytell, A. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 409.

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Effects of Thyroid Hormone and Propylthiouracil on Eruption Rate of Upper Incisor Teeth in Rats. (22125)

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Though the rat's incisor teeth continue to erupt throughout life, they are kept to a fairly constant length by the process of attrition. The rate of eruption, that is, the linear quantity by which the incisor teeth extrude into the oral cavity in a unit of time, is subject to alteration by hormonal action. Hoskins(1) found that thyroid hormone caused the incisors of newborn rats to erupt earlier than those in untreated control rats. This observation was confirmed by Karnofsky and Cronkite(2). Herzberg(3) noted that thyroid hormone caused an increase in the incisor eruption rate (I.E.R.) in adult rats; however, he offered no data to substantiate his claim. Baume, Becks and Evans(4) reported that ablation of the thyroid in adult rats produced a decrease in the I.E.R. In conformity with this view it seemed of interest therefore to extend these data by 1) quantitating the effect of thyroid hormone on the I.E.R. of adult rats and 2) testing the effect of a thyroid blocking agent, propylthiouracil (PTU) on I.E.R. The latter procedure has the advantage that it leaves the parathyroid glands in a functional state. In many of the earlier studies the parathyroids were removed indiscriminately at the time of surgical thyroidectomy.

Procedures. Twenty-three male white rats (Holtzman strain), 80 days of age, were treated as follows: 8 animals were fed ground rat chow containing 0.5% desiccated thyroid; 8 rats were fed processed chow containing 0.15% PTU and 7 animals, which served as untreated controls, were fed only ground rat chow. Four rats on PTU-feeding were shifted at the end of the fifth week to a diet of ground rat chow containing instead 0.5% desiccated thyroid for an additional 2 weeks. The I.E.R. of all animals was measured according to the method of Schour(5) once each week during the course of the experimental period. Dental roentgenograms of upper incisors of rats fed thyroid were taken after 2 weeks on the diet, and those of the animals receiving PTU were taken after 5

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TABLE I. Effect of Desiccated Thyroid and Propylthiouracil on Incisor Eruption Rate of the Rat.

Group	Wk I			Wk II			Wk III			Wk IV			Wk V		
	No. rats	I.E.R., mm/wk	Wt, g	No. rats	I.E.R., mm/wk	Wt, g	No. rats	I.E.R., mm/wk	Wt, g	No. rats	I.E.R., mm/wk	Wt, g	No. rats	I.E.R., mm/wk	Wt, g
Control	7	2.6 ± .15*	256	7	2.7 ± .33	268	7	2.6 ± .20	260	7	2.3 ± .24	273	7	2.3 ± .22	270
Thyroid, .5%	8	3.1 ± .24†	232	8	3.5 ± .24†	220	7	3.0 ± .37	193	6	3.1 ± .40	197	5	2.6 ± .36	190
PTU, .15%	8	2.4 ± .14	233	8	1.95 ± .35	237	8	1.5 ± .20	240	8	1.2 ± .39	225	8	1.2 ± .28	219

* Stand. dev.
† T test performed for difference of I.E.R. of thyroid group from control group for Weeks I and II; P = <0.01—highly significant.

weeks of treatment. These were compared with corresponding films of the controls at the respective times.

Results. Feeding of desiccated thyroid (0.5%) admixed in ground chow caused a significant increase (P = <.01) in the I.E.R. after one week, and this rose to a maximum at 2 weeks when the I.E.R. was 30% above that manifested by the control animals (Table I). Thereafter the I.E.R. declined gradually despite continued thyroid feeding and by the fifth week had returned almost to the level of that of the controls. These thyroid-fed animals manifested a severe weight loss, most of which occurred during the period of the second through the third week of treatment. From the second through the fifth weeks of the experiment 3 rats of this group died. There were no deaths in the other experimental groups during this period.

The inclusion of 0.15% PTU in the diet produced a slowing of the I.E.R. which was significant by the second week and which continued to decrease until the fourth week when it reached a minimum of 48% of that observed in the control group. The I.E.R. did not decrease further even though PTU feeding was continued through the fifth week (Table I).

When desiccated thyroid 0.5% was substituted in the diet of 4 of the above animals which had received PTU for the previous 5 weeks, the I.E.R. increased within one week to 260% over that which these same animals had registered the previous week (Table II). Thyroid feeding was continued for an additional week and the increase in I.E.R. was maintained.

The upper incisors of the animals treated with PTU for 5 weeks possessed an increased amount of dentine per unit area of incisor. (Fig. 1). This had occurred at the expense of

TABLE II. Effect of Thyroid Hormone on I.E.R. of 4 Rats Previously Treated with PTU.

Treatment	I.E.R.
PTU, 0.15% in diet, wk I-V	mm during wk V, 1.0±.24
Thyroid, 0.5% in diet, wk VI & VII	VI, 3.6±.18 VII, 3.5±.20

FIG. 1. Roentgenographs of incisors: C—Control animal. P—After 5 wk propylthiouracil feeding showing an increased amount of dentine which partially obliterates pulp chamber. T—Two wk of thyroid feeding showing enlarged pulp chamber associated with decreased amount of dentine per unit area of tooth.

the pulp chamber which was partially obliterated. Conversely the incisors of the thyroid-treated rats had large pulp chambers with a decreased amount of dentine by comparison with the films of control animals. It is to be noted that the roentgenograms were taken at the time when the maximum effects of the experimental treatment on the I.E.R. were demonstrated, *i.e.*, the second week of desiccated thyroid- and the fifth week of PTU-feedings.

Discussion. These data confirm the statement of Hertzberg that thyroid hormone increases the I.E.R. in normal rats. The response was most marked during the first two weeks of treatment and thereafter subsiding toward normal. An explanation of this phenomenon may be that in the first 2 weeks the effects on eruption are those of increased thyroid hormone action in healthy animals, whereas with continued thyroid treatment the toxic effects of the increased dosage, manifested by severe weight loss as well as the death of some of the treated animals, are noted. That the effects of thyroid hormone administration are accumulative is well known. Although the eruption rate decreases from the peak reached in the first two weeks of treatment it does not return to the rate measured in the control animals.

The increased size of the pulp chambers of the incisors of the thyroid-fed group is probably due to the increase in the I.E.R. with no apparent change in the rate of dentinogenesis. There is therefore less time for dentine to be deposited per unit area of tooth with the result that there is less encroachment upon the pulp chamber. Conversely the roentgenograms of the upper incisors of animals given PTU demonstrate an obliteration of a large portion of the pulp chamber by dentine. A likely explanation here is that while the I.E.R. is decreased the rate of dentinogenesis is apparently not affected, hence the period during which dentine can be layed down per unit area of tooth is extended. This is also in agreement with the histological evidence

from the thyroidectomized animals of Baume *et al.*(4).

The increased sensitivity of the eruptive mechanism to thyroid hormone manifested by the PTU rats is probably due to the hypothyroid state of these animals and thus the end organs which are normally affected by this hormone become more reactive.

PTU causes a chemical thyroidectomy and produces a retardation of the I.E.R. which confirms the work of Baume *et al.*(4) in demonstrating a decreased eruption rate in the thyroidectomized adult rat. Since PTU does not affect the parathyroid gland, it is evident that the reduced I.E.R. can only be due to the lack of thyroid hormone.

Conclusions. Thyroid hormone is essential for a normal rate of incisor eruption. An excess of this hormone will cause an increased eruption rate. The use of the incisor eruption rate as a tool for demonstrating hormonal activity should be considered.

1. Hoskins, M., PROC. SOC. EXP. BIOL. AND MED., 1927, v25, 55.

2. Karnofsky, D., and Cronkite, E. P., *ibid.*, 1939, v40, 568.

3. Farris, E. J., and Griffith, J. Q., *The Rat in Laboratory Investigation*, Philadelphia, Lippincott, 1949, p152.

4. Baume, L. J., Becks, H., and Evans, H. M., *J. Dental Research*, 1954, v33, 80.

5. Schour, I., and van Dyke, H. B., *Am. J. Anat.*, 1932, v50, 397.

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Relative Pathogenicity of Newly Isolated Strains of Poliomyelitis for Monkey Kidney Cells and Mice. (22126)

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Adaptation of poliomyelitis viruses to passage in mice has been accomplished only with difficulty or after laboratory manipulation of newly isolated strains(1-7). Quantitative comparisons of the pathogenicity of poliomyelitis viruses in tissue culture and in animals have usually been carried out with well adapted laboratory strains(8-11) or have been limited because of the necessary use of large numbers of monkeys(12).

The purpose of this study is to compare the quantitative relationships of the ability of freshly isolated polio virus strains to cause cytopathogenic effect in monkey kidney epithelium in tissue culture and paralysis in mice. For this purpose a number of strains of each of the three types of polio virus isolated in tissue culture from stools of patients during a national survey in 1952 was available for this study(13).

Materials and methods. Ten percent suspensions of stools were prepared in a mortar with phosphate buffered saline containing 2500 units of penicillin and 1250 μg of streptomycin per ml, clarified by centrifugation at 8,000 r.p.m. for one-half hour, and the supernates used as inocula. Primary isolations were made in cultures of monkey kidney, either in flasks of suspended tissue fragments, according to the method of Weller, Enders, Robbins and Stoddard(14), with subsequent passage in roller tubes, or directly in monocellular layer roller tubes, prepared by the method of Youngner(15). Isolates were typed in roller tubes by neutralization with specific monkey antisera. All inoculations of mice and titrations in both mice and kidney roller tubes were carried out on virus strains in the first or second tissue culture passage from the original stool. Groups of ten 4-5-week-old CFW mice were inoculated intraspinally (I.S.) with 0.02 ml, and observed for 21 days. Type 2 isolates were also inoculated intracerebrally (I.C.), in a dose of 0.03 ml, into 10 mice each, which were observed for 28 days. Wherever indi-

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TABLE I. Results of Mouse Inoculation of Polio Strains in 1st or 2nd Monkey Kidney T.C. Passage.

	Intraspinal			Intracerebral		
Type	No. tested	No. pos.	% pos.	No. tested	No. pos.	% pos.
1	51	0	0	—	—	—
2	56	45	80	56	11	20
Original stools	39	6	15	6*	1	17
3	20	0	0	—	—	—

* All 6 were positive by I.S. inoculation.

cated, tissue culture harvests were titrated by the I.S. route, using 10 mice for each serial 10-fold dilution in saline. Fifty percent end-points were calculated by the method of Reed and Muench and the results expressed as titer per 1 ml of tissue culture supernatant. Parallel tissue culture titrations were performed in monocellular layer kidney roller tubes with Type 2 strains from the same tissue culture passage material used for inoculation of mice. Tenfold serial dilutions were made in Hanks' balanced salt solution (without bicarbonate), and 0.1 ml inoculated into each of 2 tubes, which were observed for cytopathogenic effect for 7 days. Results are expressed as titer per 1 ml of fluid.

Results. Isolations in mice. A. *Types 1 and 3.* Fifty-one Type 1 and 20 Type 3 isolates were inoculated into mice I.S. In view of the entirely negative results, as shown in Table I, I.C. inoculation of mice and titrations in tissue culture was not performed. B. *Type 2.* Harvests from the first or second passage in kidney tissue culture of Type 2 viruses from 56 patients produced paralysis in mice by the I.S. route in 45 instances (80%). (Table I) The average incubation period to paralysis of the first mouse in all groups receiving the undiluted tissue culture virus was 3.8 days.

These same 56 tissue culture passage fluids were also inoculated into mice by the I.C. route and only 11 (20%) were positive. Ten produced paralysis in only one or 2 mice out of 10, and one paralyzed all 10 mice inoculated. The average incubation period to first symptoms was 7.5 days (including one group in which the single positive mouse became paralyzed on the 27th day).

Direct inoculation of stool suspensions into mice. Thirty-nine stool suspensions were tested intraspinally. As shown in Table I, direct isolation of Type 2 strains was possible with 6 (15%), although no more than one or 2 animals of 10 inoculated developed paralysis. Virus concentration in the stool suspensions was not determined by titration in tissue culture. However, no correlation was apparent between ease of isolation directly from the stools and the titer of first passage tissue culture fluid either in mice or in tissue culture. All positive isolations directly from stool were from specimens which were also positive in mice after tissue culture passage. Six stools, positive by the I.S. route, were tested intracerebrally. One produced paralysis in a single mouse. The identity of this isolate was proved by specific neutralization with Type 2 serum.

Relationship between I.S. titer and I.C. results in mice with Type 2 isolates. Table II shows a comparison of the I.S. and I.C. routes of inoculation. No fluid was positive by the latter which had not produced paralysis by the former. A correlation between I.S. titer and I.C. pathogenicity is suggested by the fact that all 8 samples with I.S. titers greater than 10^{-4} were positive by the I.C. route, whereas only one of 18 with titers less than 10^{-2} was similarly positive. The one strain that paralyzed all mice inoculated I.C. titered $10^{-5.5}$ by the I.S. route.

Relationship between titers in tissue culture and I.S. in mice. In Table III the pathogenicity for monkey epithelium cells as indicated by titer in tissue culture and for the mouse spinal cord as evidenced by the I.S. titer is compared. There appears to be no significant correlation between the two since at any level of pathogenicity for one system there is a wide spread of the titer end-points

TABLE II. Relationship between I.S. Titer and I.C. Results with Type 2 Strains.

	I.C. results	
I.S. titer	Positive	Negative
>4*	8	0
3–4	1	9
2–3	1	8
<2	1	17
0	0	11
Total	11	45

* Reciprocal of log titer/ml.

TABLE III. Comparison of Tissue Culture and Mouse I.S. Titers with Type 2 Strains.

T.C. titer	I.S. titer Neg.	<2 (undil.)	2–3	3–4	>4	Total
>7	3	2	1	0	4	10
6–7	5	7	4	7	3	26
5–6	2	7	2	3	1	15
4–5	1	2	2	0	0	5
Total	11	18	9	10	8	56

All titers expressed as the reciprocal of the log titer/ml.

by the other. Both first and second passage harvests from kidney roller tubes were tested in mice with ten strains. With 3, both were positive in high titer by the I.S. route. Of the remaining 7, 4 converted from negative to positive, but with low titers; 2 remained negative. Again, no correlation between tissue culture titer and the results in mice was demonstrable.

Relationship of clinical type of disease in patient source of virus and pathogenicity of strains. There were insufficient numbers of Type 2 virus strains from non-paralytic(11) and minor illness(8) patients to allow any significant comparison of titer results between these groups and the larger group of paralytic cases. However, while there appeared to be no difference in the tissue culture titers between these 3 groups, the strains from minor illness patients tended to have lower pathogenicity for mice than those from paralytic and non-paralytic cases.

Discussion. The entirely negative results obtained in the present study with 51 Type 1 and 20 Type 3 strains confirm the fact that the mouse pathogenicity of these immunologic types is extremely low.

Contrary to the difficulties encountered by earlier workers in adapting strains of Type 2 polio virus to mice by the I.C. route, the present study demonstrates the ease with which this can be accomplished with recently isolated strains when mice are inoculated I.S. Of a total of 56 strains tested by both routes, 20% were positive I.C., while 80% produced paralysis by the I.S. route. In a previous publication, one of the authors(16) showed a difference of approximately a log in titer between the two routes. In the present study all strains with I.S. titers greater than 10^{-4} per ml produced paralysis by the I.C. route, while only 8% of those with I.S. titers less than 10^{-4} were similarly positive.

That the high proportion of positive results reflects increased susceptibility of mice to I.S. inoculation, rather than increased concentrations of virus obtained in tissue culture, is brought out by the results of parallel titrations in the two systems. No correlation between tissue culture and mouse titer could be demonstrated. Four of five isolates in which the tissue culture titer was less than 10^{-5} produced paralysis in mice. On the other hand, of 36 fluids with tissue culture titers greater than 10^{-6}, eight were entirely negative in mice.

It is of some interest to note that direct primary isolation was possible in mice with 6 of 39 stool suspensions tested I.S., and that in one case, primary isolation was accomplished by the I.C. route. Five were obtained from paralytic cases, and one (that positive I.C.) from a non-paralytic case.

Numbers of specimens available from different classes of clinical disease were too small for significant comparisons, but the Type 2 strains isolated from those with minor illness tended to be low in their pathogenicity for mice by the I.S. route. Further investigation of this relationship between severity of clinical disease and pathogenicity must await the availability of larger numbers of strains.

Summary. Polio virus in tissue culture fluids from first or second passage in monkey kidney roller tubes was tested in mice. Fifty-one Type 1 and 20 Type 3 strains were entirely negative when inoculated I.S. Forty-five of 56 Type 2 strains produced paralysis by the I.S. route, and 11 by the I.C. route. Those having the higher titers by I.S. route tended to be positive I.C. No correlation between tissue culture titer and mouse I.S. titer was apparent. Six strains of Type 2 were isolated directly in mice from 39 stools tested.

1. Armstrong, C., *Pub. Health Rep.*, 1939, v54, 2302.

2. Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science*, 1943, v98, 452.

3. Melnick, J. L., and Ward, R., *Fed. Proc.*, 1948, v7, 308.

4. Koprowski, H., Norton, T. W., and McDermott,

W., *Pub. Health Rep.*, 1947, v62, 1467.
5. Bodian, D., PROC. SOC. EXP. BIOL. AND MED, 1949, v70, 1.
6. Li, C. P., and Habel, K., *ibid.*, 1951, v78, 233.
7. Li, C. P., and Schaeffer, M., *ibid.*, 1953, v82, 477.
8. Koprowski, H., Jervis, G. A., Norton, R. W., and Pfeister, K., *ibid.*, 1954, v86, 238.
9. Li, C. P., and Schaeffer, M., *ibid.*, 1954, v87, 148.
10. Krech, V., *ibid.*, 1954, v86, 488.
11. Wenner, H. A., *Ann. N. Y. Acad. Sci.*, 1955, v61, 840.
12. Sabin, A. B., *ibid.*, 1955, v61, 924.
13. Shelokov, A., Habel, K., and McKinstry, D. W., *ibid.*, 1955, v61, 998.
14. Weller, T. H., Enders, J. F., Robbins, F. C., and Stoddard, M. B., *J. Immunol.*, 1952, v69, 645.
15. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.
16. Habel, K., *ibid.*, 1951, v76, 357.

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Occurrence of Pantothenyl Cysteine in Natural Materials.* (22127)

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Pantothenyl cysteine (PA-cysteine)† has been recognized as an early intermediate in the biosynthesis of coenzyme A (CoA) from pantothenic acid (PA). A liver enzyme can convert PA-cysteine to pantetheine (LBF) (1). PA-cysteine has been shown to be a precursor of LBF in *Acetobacter suboxydans* and it is strongly stimulatory to this organism (2,3). It is of interest to determine whether PA-cysteine occurs naturally. Because of the need for specificity and ability to determine materials in small quantities, a bioautographic method was developed to determine various conjugates occurring in natural materials. A strain of *A. suboxydans* 621 was used. This bacterium will grow on most known PA conjugates, and is generally more sensitive to them than to free PA(2,4).

Methods. A synthetic medium previously described(5) with 2% agar was used for the bioautograph. The sterilized medium was inoculated with a heavy culture of *A. suboxydans at* 40° and poured into a sterile container to a depth of 6-8 mm. This container consisted of a 19 x 50 cm glass plate, which rested within a stainless steel frame 15 mm high. After agar had solidified, a developed paper chromatogram of PA conjugates was sterilized by treatment with ether, and then spread on agar surface. After 10 minutes, the paper was peeled off, agar plate covered with a sterile 19x50 cm glass plate and incubated at 30° for 24 hours. The top plate was then removed and agar surface sprayed lightly with a mixture of PA (10 γ/ml) and yeast extract (100 mg/ml). The glass plate was restored and the whole assembly again incubated at 30° for 24 hours. Aseptic conditions were preserved throughout. The bioautograph was then observed against a background of diffuse light. The PA-yeast extract mixture was introduced because *A. suboxydans* otherwise grows very slowly under conditions employed. This mixture stimulated growth throughout the entire agar plate but it exerted its greatest effect in areas where growth had already been initiated, *i.e.*, where prior contact had been made with the PA conjugates on the developed chromatogram. *A beef liver* preparation was used as source of naturally occurring PA-cysteine. It was prepared as follows: 15 g of cold beef liver were ground in a Waring blendor with 50 ml

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† The following abbreviations will be used throughout this paper: pantothenic acid = PA; coenzyme A = CoA; pantetheine = *Lactobacillus bulgaricus* factor = LBF; pantetheine-γ-phosphate = LBF-γ-phosphate; pantothenyl cysteine = PA-cysteine.

of ice water. This was poured into 50 ml of boiling water and the liquid decanted from the coagulated protein. Trichloroacetic acid was added to 1% concentration and the solution centrifuged. This yellow liquid was then extracted with ether in a liquid-liquid extractor, and the aqueous layer again centrifuged. The supernatant liquid was passed through a Dowex 1 column (2% cross linked), which had previously been charged with 3 M ammonium formate and washed with water. The column was eluted with formate buffer, prepared by adding 90% formic acid to 0.3 M ammonium formate to pH 3.5. The initial 20 ml of eluate (which was presumably free of coenzyme A(6) as well as other phosphorylated PA derivatives) was selected as the source of natural material used on paper chromatograms. Formate was eliminated by adding excess of HCl and concentrating eluate over KOH in vacuum desiccator. This eluate was reduced, first with Zn-HCl, then with H_2S at pH 7.5, to insure that all PA conjugates present were in the thiol form. About 20 μl of solution were then placed on Whatman No. 1 paper and chromatographed by the ascending technic. 1 γ of PA, LBF, CoA, LBF-γ-phosphate, and PA-cysteine each were used in 20 μl solution, to give zones of reference on chromatogram. To make sure that no reoxidation to the disulfide took place, some chromatograms were run under a nitrogen atmosphere. Results were identical to those obtained with chromatograms run in air.

Results. This method is adequate for detection and identification of low concentrations of PA conjugates. PA, CoA, and PA-cysteine have been detected at 0.2 γ. Since *A. suboxydans* did not grow on PA phosphates (7), it is not expected that this method will detect all possible conjugates of PA.

Table I summarizes the R_f values of standard conjugates and liver preparation in a number of solvent systems. Pyridine-water-isopropyl alcohol (1:1:1), phenol-water (4:1), or acetone-water-urea (59.7:39.8:0.5) proved to be good systems for distinguishing PA-cysteine from other conjugates tested. Two-dimensional chromatograms using these solvents disclosed three zones of PA activity. One of these corresponded to the free vitamin, another to PA-cysteine. The third zone did not consistently appear throughout the many experiments performed, and it may be present in a much lower concentration. Using butanol-water, it was not possible to resolve the liver preparation sufficiently to demonstrate PA-cysteine.

TABLE I. R_f Values of Some PA Conjugates, Determined by Bioautographic Technic.

	Solvent			
Compound	Phenol-water (4:1)	Butanol sat. with H_2O	Pyridine-isopropyl alcohol-water (1:1:1)	Acetone 59.7%, water 39.8%, urea 0.5%
PA-cysteine	.48	.17	.75	.76
PA	.75	.35	.80	.66
CoA	.20	0		.16
LBF-γ-phosphate	.94	.81	.96	.46
LBF	.90	.94	.90	.92
Beef liver preparation	.73	0	.80	.70
	.61	.29	.73	.63
	.50		.60	.54

Summary. A bioautographic technic has been described for the detection of PA conjugates in natural materials. 0.2 γ of PA, PA-cysteine and CoA can be detected. The occurrence of PA-cysteine in beef liver is indicated by this method.

1. Hoagland, M. B., and Novelli, G. D., *J. Biol. Chem.*, 1954, v207, 767.

2. King, T. E., and Cheldelin, V. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 591.

3. Brown, G. M., and Snell, E. E., *J. Am. Chem. Soc.*, 1953, v75, 2782.

4. ———, *J. Bact.*, 1954, v67, 465.

5. King, T. E., and Cheldelin, V. H., *ibid.*, 1950, v59, 229.

6. Novelli, G. D., and Schmetz, F. J., *J. Biol. Chem.*, 1951, v192, 181.

7. King, T. E., and Strong, F. M., *ibid.*, 1951, v191, 515.

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Analgetic Property of Vitamin K. (22128)

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(Introduced by A. J. Goldforb.)

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Vitamin K stimulates the liver in order to produce prothrombin. Insufficient amounts of vit. K produce insufficient amounts of prothrombin. From this arises one of the conditions for haemorrhagic diathesis. Vit. K is administered to prevent haemorrhagic diathesis following hypoprothrombinemia, after surgical intervention or, prophylactically, prior to operations.

The other properties of vit. K and Synkavit (diphosphoracid-ester of 2-methyl-, 4-naphtohydrochinon) which is pharmacologically identical with vit. K, have not yet been registered. Vit. K possesses, however, a strong analgetic property which has been observed in patients(1) and experimentally proved in animals(2).

We investigated the analgetic property of vit. K on mice according to the Wolfe-MacDonald method(3), which deals with the registration of thermoalgesia, as follows:

A glass vessel with double walls between which there is water at a constant temperature of 122°F (50°C) serves for testing the susceptibility of an animal to temperature. If the mouse is put into such a vessel, it will lick its paws after a certain time to mitigate the pain caused by the temperature at the bottom of the vessel. The time elapsing from the moment when the animal is put into the vessel to the moment of licking its paws is measured by chronometer. This period indicates the susceptibility of the animal to pain caused by warmth.

The normal reaction of licking in a series of mice, an average of 9.9 sec. served as control time. The mice were then given vit. K. We took measurements 4 times *i.e.* 30 sec., 1½ hr, 2½ hr, and 3½ hr following administration of vit. K. The results are shown in Fig. 1.

Vitamin K has a decidedly thermoanalgetic property, since the reaction to pain occurs much later than in the control mice. Whereas the reaction took place after 9.9 sec. in the control mice, the reaction in mice which were administered 2 mg/kg of weight of vit. K occurred after 17 sec. and in mice which were given 4 mg/kg of weight of vit. K after 16.3 sec. Increase of the amount of vit. K does not promote the thermoanalgetic effect but lessens it slightly.

To evaluate these results we investigated the thermoanalgetic effect of morphine by the same method. A series of mice were injected with 3 mg/kg of weight of morphine intraperitoneally and another series with 6 mg/kg of weight. The results are given in Fig. 2.

The results of the thermoanalgetic effect of vit. K in doses of 2 mg/kg of weight and 4 mg/kg of weight have been recorded. The thermoanalgetic effect of vit. K is even stronger than that of morphine. Although the maximal effect of morphine occurs immediately after administration the maximum with vit. K occurs toward the end of the experiment. The reason is the rapidity of resorption. Watery solution of

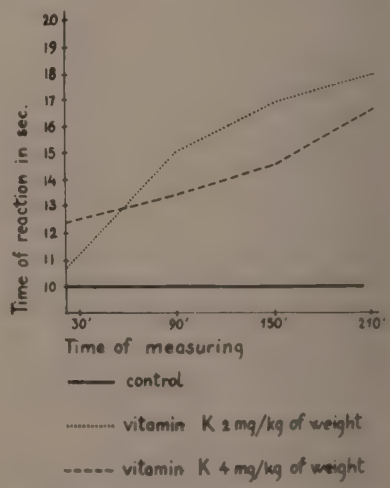

FIG. 1. Effect of Vit. K by normal mouse.

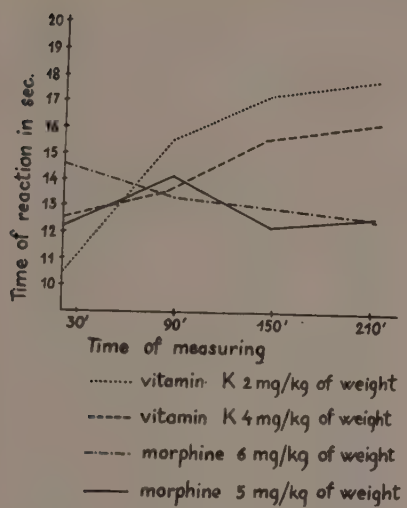

FIG. 2. Effect of Vit. K and morphine.

morphine administered intraperitoneally is readily resorbed while the oil solution of vit. K administered orally is resorbed much slower.

It is known that some substances may elicit the analgetic effect in such a way as to elicit directly or indirectly the mobilisation of cortisone from the root of the suprarenal gland through the hypophysis(4,5). Hench(6) has proved that cortisone has a highly analgetic effect and Jakob(7) has shown that morphine owes its analgetic property to the fact that it may mobilize cortisone. We therefore investigated whether vit. K can mobilize cortisone or some other hormone of the suprarenal gland. We have carried out the above described experiments on suprarenalectomised mice. The results are shown in Fig. 3.

The thermoanalgetic property of vit. K must not be ascribed to its ability to mobilize considerable amounts of cortisone in the organism since the suprarenalectomised mice showed the same reaction as normal mice. From these results we can conclude that the thermoanalgetic property of vit. K may be based on its depressive action either on the cortical or subcortical centres for pain.

To confirm our supposition that vit. K acts on certain areas of the central nervous system in an inhibiting way, we have investigated the registration of mobility according to Kosak's method(8) on a series of mice which were administered vit. K. Kosak's method seemed most satisfactory to us since it secures free movements and registers even the slightest movements. The principle of the method is the following:

The mouse is put into a vesel that is floating on water like a labile boat. All movements are transferred to the vessel and from the vessel recorded on a kymograph. From the frequency of the movements and the size of the registered curves we may deduce objective conclusions on the excitement or immobility of the animal. The results of these experiments are shown in Figs. 4 and 5. Fig. 4 shows the kymographic curve of the mouse which got 4 mg/kg of weight of vit. K and Fig. 5 shows the kymographic curve of a suprarenalectomised mouse given 4 mg/kg of weight of vit. K. From both figures the pronounced sedative and calming effect of vit. K is shown.

We also investigated whether vit. K had an antagonistic property against shock provoked with pentazol and whether it had any influence on the electroepileptic shock.

In a control series the mice were administered 75 mg/kg of weight of pentazol intraperitoneally. All mice displayed typical pronounced convulsions. In a second control series the mice were given 100 mg/kg of weight of pentazol. All mice died shortly after.

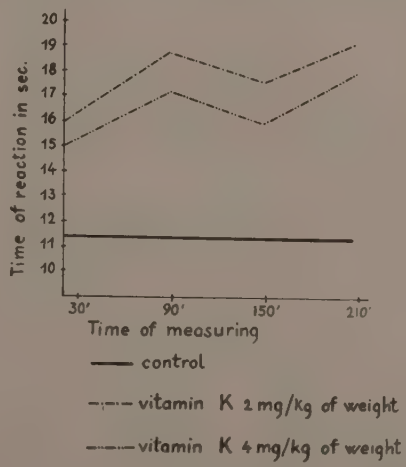

FIG. 3. Effect of Vit. K by suprarenalectomised mouse.

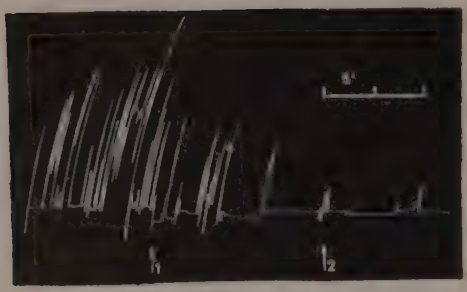

FIG. 4.

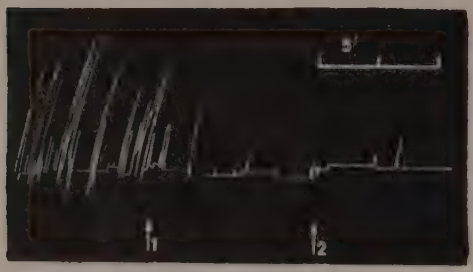

FIG. 5.

Owing to these control results a series of mice were given first 4 mg/kg of weight of vit. K and later 75 mg/kg of weight of pentazol. A second series of mice were first given 4 mg/kg of weight of vit. K and then 100 mg/kg of weight of pentazol. Vit. K had no effect either on the convulsions or on the lethality.

Finally we investigated whether vit. K had any influence on electroshock. The experiments showed that vit. K had no influence either on the intensity or on the duration of the electroshock. We have therefore concluded that vit. K has no depressive action on the motoric sphere of the brain or on the subcortical tonic centers.

We tested the analgetic property of vit. K in 115 patients with inoperative carcinomas or sarcomas with multiple metastases. All of them were given daily morphine and barbiturates against intense pain prior to initial administration of vit. K. We stopped administering morphine and barbiturates and gave daily injections of 20-30 mg of vit. K. Full analgesia was produced in 95 patients *i.e.* in 75%. In the remaining 20 patients the analgetic effect was either slighter or there was none at all.

Conclusion. 1. Vit. K has shown a considerable thermoanalgetic effect that is also evident in suprarenalectomised animals. This effect is stronger than the thermoanalgetic effect of morphine. 2. Vit. K has shown a strong sedative effect not being due to the mobilisation of the hormones of the suprarenal glands. 3. Vit. K has no depressive property on the cortical or subcortical motor-centers. 4. In patients with inoperative carcinomas and sarcomas with metastases and severe pains, vit. K showed such a pronounced analgetic property that we could cease administration of morphine and barbiturates. The complete analgetic effect was obtained in 73% of cases.

1. Kubovic-Prazic, *Lijecnicki Vjesnik,* Zagreb, Yugoslavia, 1954, v76, 1.

2. Atanackovic, *Makedonski medicinski pregled,* Skopje, Yugoslavia, 1954.

3. Wolfe-Mac Donald, *J. Pharmacol.,* 1944, v80, 300.

4. Cheymol, *Actualities Pharmacologiques.* II Serie. Masson, Paris, 1950.

5. Frommel, *Arch. Int. Pharmacodyn.* 1952, v92, 129.

6. Hench, *Proc. Staff. Meet. Mayo Clinic,* 1949, v24, 181.

7. Jakob, *Arch. Int. Pharmacodyn.,* 1952, v90, 30.

8. Kosak, *Acta Pharmacol. Yugoslav.,* 1954, v2, 83.

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Anticonvulsant Threshold of CO_2 in Oxygen under High Pressure. (22129)

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Taylor(1) found that addition of small amounts of CO_2 to the breathing mixture hastened the onset of convulsions under high oxygen pressure. Chapin(2) in determining CO_2 toxicity under OHP found no convulsions when mice were subjected to 304 mm Hg $^{P_I}CO_2$ and 2890 mm or 6080 mm PO_2. This disparity suggested that CO_2 may enhance convulsions only in low concentrations. If this is true it should be possible to determine that tension at which CO_2 becomes anti-convulsant.

Method. A high pressure gas chamber with volume about 1.2 liters was constructed from heavy 4 inch pipe closed at one end. The other end contained a thick lucite window and screwed onto the nipple. A heavy rubber gasket sealed the chamber when tight. Two ports were fitted into the nipple to facilitate introduction and expulsion of gas. Test animals were placed in the chamber which was then closed and ventilated with the test gas. When air had been expelled, exit port was closed and pressure increased to desired value.

Mice were exposed to gas mixtures containing 27, 61, 69, 97, 101, 120, 125, 132, and 165 mm $^PCO_2$ each in 3800 mm PO_2 Exposure time to onset of first convulsion was recorded in those cases where a full tonic convulsion occurred.

Results. These experiments confirm the reported enhancement of onset of convulsions by low CO_2, but in addition shows that the time by which convulsions are hastened is about the same when the $^PCO_2$ is between 27 mm and 120 mm Hg.

The tension at which only half of the mice convulse and the other half goes on to die without convulsion is about 120 mm $^PCO_2$. Table I shows the tabulated results of these experiments.

TABLE I. Influence of CO_2 on OHP Convulsions and Convulsion Time. $PO_2 = 3800$.

$^PCO_2$	No. used	No. convulsing	Min. exposure before convulsions
0	15	15	21 (10-35)
27	4	4	8 (6- 9)
61	15	15	8 (6-11)
97	3	3	12 (10-14)
101	4	4	9 (8-11)
120	10	5	9 (8-10)
125	4	0	—
132	4	0	—
165	9	1	10

Discussion. This study confirms Taylor's (1) findings that: 1) Carbon dioxide hastens onset of OHP convulsions, 2) within certain limits tension of CO_2 used to shorten convulsion time, appears to be immaterial.

It further appears that CO_2 hastens onset of OHP convulsions in one range and inhibits them in another. The division between these ranges appears to be about 120 mm $^PCO_2$. This value falls slightly below that found by Chapin(4) to produce maximal ventilatory response in the hamster and may be related to CO_2 tension at which anesthetic effects are first manifested.

Taylor did not mention failure to convulse in any of his 20 trials although he exposed one cat to 12.2% CO_2 at 55 lb pressure. (He did not specify whether this is gauge or total pressure. Neither did he specify whether this was the pressure due to O_2 and CO_2 together or O_2 alone. If it is total pressure and due to both gases, the $^PCO_2$ was 347 mm. If it is gauge pressure and due to both gases, the $^PCO_2$ was 439 mm.) This is well within the range for which CO_2 was found by this study to be anticonvulsant.

Stein and Sonnenschein(3) contended that they were unable to confirm the potentiating action of CO_2 on OHP convulsions in cats even though they used CO_2 as low as 1%. Their gauge pressure appears to have been either 75 or 90 lb. The $^PCO_2$ at which they failed to get convulsions but only depression

* The author wishes to state his appreciation for the technical assistance of C. and N. Morris, E. Lappat, and I. Miller.

may be calculated at 46 or 54 mm. This is far below the anti-convulsant threshold determined in mice by this study.

Summary. 1. A series of 68 mice was exposed to 5 atmospheres of oxygen in a small pressure chamber. Some of these received pure oxygen while others received mixtures of oxygen diluted with varying quantities of CO_2. All mice receiving only pure oxygen convulsed. All mice receiving less than 120 mm $^{P}CO_2$ in OHP convulsed. One half of the mice receiving 120 mm $^{P}CO_2$ in OHP convulsed. The other half went on to die without convulsions. Only one out of 17 mice receiving over 120 mm $^{P}CO_2$ convulsed. 2. Mice receiving oxygen diluted with CO_2 convulsed sooner than mice receiving pure oxygen. Amount of shortening of convulsion time did not appear to be dependent upon amount of CO_2 diluting the oxygen as long as $^{P}CO_2$ lay in the range of 27-120 mm. Both of these findings confirm those of Taylor(1). The tension at which CO_2 becomes anticonvulsant appears to be about 120 mm. or about 16% of 1 atmosphere.

1. Taylor, H. J., *J. Physiol.*, 1949, v109, 272.
2. Chapin, J. L., AF Tech Rep., in press.
3. Stein, S. M., and Sonnenschein, R. R., *J. Aviation Med.*, 1950, v21, 401.
4. Chapin, J. L., *Am. J. Physiol.*, 1954, v179, 146.

Received October 18, 1955. P.S.E.B.M., 1955, v90.

Effects of Altitude, Acidosis, and Alkalosis upon Hypoxic Threshold.* (22130)

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(Introduced by C. A. Maaske.)
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Hoff and Breckenridge(1) have contended since acclimatized subjects exhibit hyperventilation at all altitudes above sea level that in acclimatized breathing there is no anoxic threshold. Rahn and Otis(2) on the other hand suggest that the alveolar $^{P}CO_2$ begins to fall (and the ventilation begins to rise) in all residents of altitude when the $^{P_A}O_2$ is lowered to about 100 mm Hg.

This study was undertaken to determine whether there was or was not a hypoxic threshold for altitude dwellers, if so where it was, and whether it was the same no matter what altitude was represented.

Method. Experiments were carried out on 6 subjects, residents of Denver, Colo. (5300 feet) and on 5 others, 4 of whom had spent 2 months and one who had spent 1 month living on Mt. Evans, Colorado (14,150 feet). Subjects rested on a cot throughout experiment. After a 10 minute period to bring them to a resting state they breathed a gas mixture containing 50% oxygen in nitrogen so the ventilation changes incident to breathing oxygen mixtures described by Chapin(3) would not be introduced into the critical phase of experiment. Following this phase subjects immediately switched to rebreathing from calibrated Collins 13.5 L recording spirometer equipped with soda lime CO_2 absorber and initial gas mixture of 50% O_2 in N_2 at Mt. Evans and 35% O_2 in N_2 at Denver. By this method inspired $^{P}O_2$ was gradually reduced. Terminal gas (Scholander) analyses showed no CO_2 in spirometer at end of experiment. Spirometer O_2 was analysed each minute by pulling small samples through a Beckman infrared oxygen analyzer. The point at which ventilation began to increase was taken as hypoxic threshold.† Since, in some respects, chronic NH_4Cl ingestion simulates altitude acclimatization, we thought that testing the effect of NH_4Cl on hypoxic threshold would be interesting. Five of the Denver subjects ingested 6 g of NH_4Cl

* This study was supported in part by funds provided under contract AF 18(600)-1257 with the USAF School of Aviation Medicine, Randolph Field, Texas.

TABLE I. Hypoxic Thresholds in Various Situations.

Situation	Tracheal $^{P}O_2$ at hypoxic threshold (mm)	Range	No. of subjects
Mt. Evans	179	174 -182.5	6
Acidosis at Denver	164	153 -185	5
Normal at Denver	127	121 -131	5
Alkalosis at Denver	107	103.5-110	3
Normal at sea level	93	(Rahn and Otis)	

daily for 5 days, were tested as described above, and showed elevation in hypoxic threshold to 164 mm. Following this finding it was natural to test the effect of bicarbonate. This was done by 3 subjects who ingested 15-30 g $NaHCO_3$ per day for five days and exhibited an average hypoxic threshold drop to 107 mm which is close to that exhibited by normal subjects acclimatized to sea level.

Results. A definite threshold for Denver residents was found at about 127 mm $^{P_I}O_2$. This suggests that most Denverites have slight hypoxic drives since average Denver tracheal air $^{P}O_2$ is about 122 mm. Mt. Evans residents also exhibited thresholds but these averaged 179 mm $^{P_I}O_2$. Since sea level subjects are believed by many to reach the hypoxic threshold only when inspired oxygen falls to 12 to 14%, there appears to be an orderly progression of hypoxic threshold with altitude.

Table I shows the mean hypoxic thresholds in each of 4 experimental situations together with the ranges and number of subjects. In addition a sea level figure from Rahn and Otis is included.

Discussion. One might argue, since the hypoxic threshold varies directly with altitude, that it varies inversely with the $^{P}O_2$ at which one is acclimatized. However, since NH_4Cl ingestion at Denver results in hyperventilation and slightly elevated $^{P_A}O_2$ and at the same time increases the hypoxic threshold, this thesis would not appear to be tenable. It might be further argued that pH determines the threshold. However, since altitude acclimatization probably results in slightly elevated pH while NH_4Cl administration lowers pH somewhat and since they both elevate the hypoxic threshold, this thesis seems untenable. It seems more likely that the hypoxic threshold varies with the resting $^{P}CO_2$ level or some function thereof. $^{P}CO_2$ is low in individuals acclimatized to altitude and in those in chronic NH_4Cl acidosis. Both of these groups exhibit high thresholds. $^{P}CO_2$ is higher in individuals acclimatized to sea level than in Denverites and it is also high in Denverites receiving bicarbonate. Both of these groups show low hypoxic thresholds.

Still further possibilities remain. Acclimatization of the chemoreceptors to the bicarbonate level of the arterial blood may govern the threshold. The chemoreceptors may be involved only indirectly and the level of their response to anoxia may be set by the $^{P}CO_2$ or long term bicarbonate level of the respiratory center. In some way the volume of ventilation, apart from CO_2 level, might set the threshold. On the other hand, if it is CO_2 or bicarbonate level to which one is acclimatized which determines the threshold for hypoxic response, this test might be a more sensitive measure of the extent of CO_2 acclimatization than those we now have.

Fig. 1 shows a plot of these points on a Fenn CO_2-O_2 diagram. Inspired tensions have been converted to probable alveolar values and alveolar hypoxic threshold CO_2 tensions have been calculated assuming a steady state and assuming that high oxygen depresses breathing in subjects acclimatized to altitude (3). A hypoxic threshold line has been drawn connecting the threshold values. This line confirms the suggestion that a resident of about four thousand feet would be living at his hypoxic threshold. However an alkalotic individual would be above and an acidotic individual would be below the threshold and the latter would be under some hypoxic ven-

† Although it would be conventional where the threshold occurs at a higher O_2 tension to refer to it as a reduced hypoxic threshold (lower intensity of a liminal stimulus), we feel such usage would be more confusing than to refer to the threshold in terms of the oxygen tension at which it occurs. The above situation, therefore, would be called an increase in hypoxic threshold.

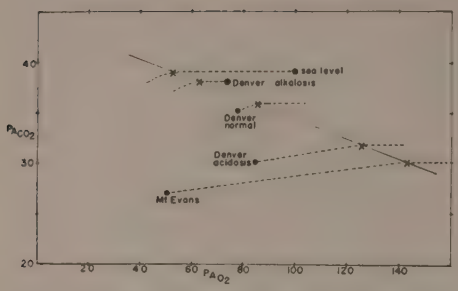

FIG. 1. Hypoxic threshold line is the solid line drawn between probable hypoxic thresholds of residents at sea level, Denver, Mt. Evans, and of Denver residents who are acidotic and alkalotic. Dashed lines indicate probable alveolar gas changes resulting from gradual decreases in alveolar oxygen tensions. When the threshold line is crossed, breathing increases and $^{P_A}CO_2$ decreases.

tilatory drive.

Summary. 1. Inspired oxygen tensions which produce the first discernible increases in ventilation (hypoxic thresholds) were measured in subjects acclimatized to Mt. Evans, Colo. (14,150 feet) and to Denver, Colo. (5300 feet). In addition thresholds were measured in subjects living in Denver who were in chronic metabolic acidosis (NH_4Cl) and in chronic metabolic alkalosis ($NaHCO_3$). 2. Mt. Evans subjects showed an average threshold of 179 mm tracheal $^{P}O_2$ and those at Denver 127 mm. Reports in the literature pinpoint the threshold for sea level dwellers at about 93 mm. Furthermore the threshold for Denverites ingesting NH_4Cl was 163 mm and for those ingesting $NaHCO_3$ was 107 mm. 3. Since NH_4Cl changed $^{P_A}O_2$ and pH in the opposite direction from altitude but changed the hypoxic threshold in the same direction, these agents were ruled out as threshold governors. It was suggested that probably the hypoxic threshold is a function of either the resting $^{P}CO_2$ or the bicarbonate level to which the individual had acclimatized.

1. Hoff, H. E., and Breckinridge, E. C. Regulation of Respiration in Fulton's *Textbook of Physiology*, 17th ed., W. B. Saunders Co., 1955.

2. Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1949, v157, 445.

3. Chapin, J. L., *J. Aviation Med.*, 1954, v25, 500.

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Prevention and Treatment of Motion Sickness by Intranasal Medication. (22131)

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Rapid absorption of hyoscine hydrobromide (scopolamine) after intranasal instillation has recently been reported(1,2). This route of administration combines simplicity of self-medication with rapid drug absorption. It has the further advantage of circumventing the oral route when nausea, psychic factors, or lack of cooperation make swallowing difficult. This technic should be especially valuable in prevention and treatment of motion sickness. Its effectiveness against swing- and airsickness is the subject of this report.

Experimental. Scopolamine Spray and Swing Sickness. Healthy, male adults ranging in age from 17 to 24 years (average 19 years) were used as subjects. The experimental group were given varying amounts of scopolamine in each nostril by a plastic spray bottle† and the remainder were given saline in a similar manner. Exact amount of fluid instilled was determined by loss in weight of spray bottle. Thirty minutes after spraying, the subject was placed in the carriage of a motor-driven swing and swung through an arc of 120° for 20 min-

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† We acknowledge a generous supply by the Doho Chemical Corp.

utes, unless vomiting occurred earlier. Radius of swing was 14 feet and frequency of swingings 14 per minute. Data on occurrence of side effects was obtained from questionnaires distributed upon completion of swinging. Scopolamine was dissolved in 0.9% NaCl solution immediately before use. Originally, it was planned to spray approximately 0.3 mg of scopolamine into each nostril. Therefore, a solution containing 3 mg/ml was prepared and approximately 0.1 ml sprayed into each nostril. The volume sprayed varied considerably and an occasional subject received 1 to 1.5 mg of scopolamine. Dosage level was, therefore, reduced to 2 mg/ml to provide a greater margin of safety. As evident from Table I significant protection was afforded although the average dose was only 0.43 mg ($\sigma = 0.22$) compared with 0.65 mg to 1 mg ordinarily given by mouth. At this dose level, the only side effects markedly increased were those of dizziness, which rose from 23% to over 45%, and dry mouth which was more than twice that of control group. There was no significant increase of drowsiness or blurred vision. Other side effects such as headache, fatigue, nervousness, and sweating were uniformly less than the incidence among the control group. The high percentage of mild dizziness after scopolamine (despite the relatively small dose administered) was disturbing. Therefore, to increase further the margin of safety, scopolamine was prepared in a concentration of 1 mg/ml and administered as before. With this solution, an average dose of 0.26 mg ($\sigma = 0.14$) of scopolamine was sprayed. The number vomiting was slightly less than in the control group but the difference was not statistically significant. Since the detergent sodium lauryl sulfate (Duponal C) increased the absorption of scopolamine in a nasal spray(1) it was added (0.01%) to the saline solution (0.9%) containing 1 mg/ml of scopolamine. As is evident from Table I this preparation sharply decreased the incidence of vomiting from 15.3 to 5.9%. Incidence of dry mouth was greater than normal, but no other significant increases in side effects were apparent. Incidence of headaches, sweating, and fatigue was reduced. The average dose of scopolamine administered was 0.31 mg ($\sigma =$ 0.12). This was slightly higher than the amount sprayed when Duponal was not employed at the 1-mg dose level.

TABLE I. Effectiveness of Scopolamine against Swing-Sickness when Administered as Nasal Spray.

	No. of subjects	Avg dose, mg	Vomit, %	Protection,† %
Saline control	47		14.9	71.8
2 mg S‡	47	.43	4.2*	
Saline control	94		13.8	23.2
1 mg S	94	.26	10.6	
Saline-Duponal control	85		15.3	61.5
1 mg S (0.9%)—Duponal (0.01%) sol.	85	.31	5.9*	

* $P = \leq 0.05$.

† $\dfrac{\text{\% of vomiting placebo} - \text{\% of vomit. medication}}{\text{\% of vomiting placebo}}$.

‡ S = Scopolamine/ml in saline.

Scopolamine Spray and Airsickness. The effectiveness of intranasal administration in preventing swing sickness suggested that it be tried therapeutically as well. For this purpose, our subjects were exposed to an hour's flight of standardized turbulence in C-47 (DC-3-type) aircraft. Procedure was the same as previously reported(3) in which the route, altitude, and maneuvers were standardized as rigidly as possible to insure reproducible flights. Originally, the subjects were given the plastic spray bottles containing either control saline-Duponal solution or freshly prepared scopolamine (2 mg/ml) in this solution. Each person was instructed to use the spray as soon as he felt nauseated. It rapidly became apparent, that the subjects were reluctant to medicate themselves since to do so constituted an admission of nausea. Consequently, very few utilized the medication despite obvious distress and subsequent vomiting in many cases. To obviate this difficulty, observers sprayed each subject intranasally 15 minutes after take-off. This time was chosen since in scores of previous flights vomiting rarely occurred earlier, although vasomotor disturbances and nausea were already evident. The spray bottles were weighed before and after use to determine the quantity lost. The subjects were observed for remainder of test period and the incidence of side effects was obtained by questionnaire, as

TABLE II. Therapeutic Effectiveness of Nasal Administration of Scopolamine.*

Dose of scopolamine	No. of subjects	Vomit, %	P†	Protection, %
0 (saline)‡	79	44.3	—	—
.80 ($\sigma = 0.50$)‡	79	22.6	.01	48.6
0 (saline)§	89	28.1	—	—
.2 §	90	17.8	.10-.20	36.6
.4 §	90	15.6	.05	44.5
.6 §	90	10.0	<.01	65.0

* Administered 15 min. after take-off.
† Saline vs medication.
‡ Nasal spray.
§ Nose drops.

already described. The results are summarized in Table II. As can be seen, the scopolamine spray significantly reduced the incidence of vomiting. Since medication was not administered until 15 minutes after flight had begun and the average time of vomiting was 30 to 35 minutes, it is apparent that absorption and action of scopolamine by this route is extremely rapid. The incidence of blurred vision, drowsiness, and dry mouth was increased in the medicated group, although not to the significant level. Sweating was significantly reduced ($P = 0.01$) among those receiving scopolamine.

Scopolamine Nose Drops and Airsickness. Despite careful selection of the spray bottles and indoctrination in their use, the variation in amount of drug given was surprisingly great, ranging from less than 0.10 mg to more than 1.5 mg ($\sigma = 0.50$). To obtain more exact dosage response data, the experiment was repeated utilizing nose drops rather than the spray. Again, 15 minutes after take-off, 0.2 ml of solution was instilled into each nostril. Varying quantities of scopolamine were dissolved in 0.9% NaCl containing 0.01% Duponal so that total dose administered was 0.2, 0.4, or 0.6 mg, respectively. A control group received the Duponal-saline solution alone. The results are given in Table II. Marked protection was afforded by 0.4 and 0.6 mg of scopolamine and apparently some protection even by 0.2 mg. Significant side effects were confined to blurred vision, dry mouth, and sweating. No changes were observed with 0.2 mg of scopolamine, except for a decrease in incidence of sweating. At the 0.4 mg level, blurred vision was reported, and with 0.6 mg all 3 side effects were accentuated. A slight, but nonsignificant, increase in drowsiness was also reported.

Effectiveness of Scopolamine by Various Routes Against Airsickness. Another practical means for self-administration of drugs is by the sublingual route. Many drugs have been given in this fashion and absorption is known to be rapid. It was of interest, therefore, to compare effectiveness of scopolamine by this route with that after intranasal instillation. Volunteers were again subjected to a period of standardized turbulence in military aircraft before receiving medication. Eighteen minutes after take-off, subjects received a placebo capsule taken by mouth, or 0.65 mg of scopolamine by mouth, or as sublingual tablet. Two minutes later, a fourth group received the same amount of scopolamine in the saline-Duponal solution as nose drops. The 2-minute interval permitted dissolution of the sublingual tablet. Regular 0.01 grain hypodermic tablets were used for this purpose. These small tablets were placed under the tongue and the subject cautioned not to swallow. They were found to dissolve well within the 2-minute period. Exposure time prior to medication was lengthened (20 vs. 15 min) over that in earlier trials to provide a more rigorous test for the drug. As before, scopolamine nose drops markedly lowered the incidence of vomiting (Table III), whereas the drug was completely ineffective when given orally or sublingually. The rapidity of its absorption through the nasal mucosa is also reflected by side effects recorded in Table IV. Nose drops produced highly significant increases ($P = 0.01$) in dizziness, drowsiness, and dry mouth, as well as a significant decrease in incidence of headaches. The only other significant change from the control group was a moderate increase ($P = 0.05$) in

TABLE III. Route of Administration on Effectiveness against Airsickness.

	Dose, mg	Route	No. of subjects	No. of vomit	Vomit, %
Placebo		Oral	84	29	34.5
Scopolamine	.65	"	85	30	35.3
	.65	Sublingual	82	26	31.8
	.65	Intranasal	82	13	15.9

TABLE IV. Side Effects of Scopolamine by Various Routes.

Dose, mg	Route	Dizzy	Drowsy	Dry mouth	Headache
		%			
0	Oral	16.5	41.2	9.4	22.4
.65	"	23.5	47.1	12.9	16.5
.65	Sublingual	22.4	40.0	20.0*	18.8
.65	Intranasal	45.9†	69.4†	47.1†	8.2†

* P = .05. † P = .01.

dry mouth among those receiving scopolamine sublingually.

Discussion. It is apparent from these results that intranasal medication is feasible in the prevention and treatment of motion sickness. The rapid action of scopolamine by this route was the most striking feature. In the aircraft, vomiting was largely prevented despite the short period available for its action on an already nauseated individual. Since the drug was not given until 15-20 minutes after take-off and the test period was only 60 minutes, a maximum period of 40-45 minutes was available for absorption and action. As expected because of the short period allowed for absorption, scopolamine by mouth was completely ineffective. Ineffectiveness by sublingual route indicates that absorption here, too, is slower than from the nasal mucosa.

Administration as a spray would seem the simplest technic for intranasal instillation. The marked variation in the quantities nebulized, however, makes this procedure unreliable at present. Small, disposable spray bottles holding only a single dose would obviate this difficulty, if their production is feasible. For the present, nose drops, although slightly more difficult to administer are preferable since their dose can be more accurately controlled.

Summary. Scopolamine in small doses (0.3-0.4 mg) given intranasally by spray 30 minutes prior to exposure, exerted significant protection against swing sickness. During actual flight testing, addition of a surface active agent (sodium lauryl sulfate-Duponal C) increased its effectiveness. Nasal instillation to subjects 15-20 minutes after take-off sharply reduced the incidence of vomiting from airsickness during subsequent 40-45 minutes. Oral and sublingual administration under these conditions were ineffective. Considerable variations in the drug instilled resulted when given by spray. The use of nose drops allowed more accurate medication. The significance of this mode of administration for treating motion sickness is discussed.

The invaluable assistance of Major H. J. Garza, Capt. W. L. Simpson, J. E. Hammer, A. P. Thiesen, and S/Sgt. E. C. Stevens of the Flight Section, School of Aviation Medicine, USAF, in providing a standardized flight for these studies is gratefully acknowledged.

1. Tonndorf, J., Hyde, R. W., Chinn, H. I., and Lett, J. E., *Ann. Oto. Rhin. and Laryng.*, 1953, v62, 630.

2. Hyde, R. W., Tonndorf, J., and Chinn, H. I., *Ann. Otol. Rhin. and Laryng.*, 1953, v62, 957.

3. Chinn, H. I., Milch, L. J., and Dugi, A. J., *J. Appl. Physiol.*, 1953, v6, 257.

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Changes in Adrenal Medulla Following Treatment with an ACTH-Preparation Containing Carboxymethylcellulose.* (22132)

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Several investigators believed to have produced changes in the adrenal medulla by treatment with certain pituitary preparations. It was thought, for instance, that injections (1) or even oral administration(2) of a special "medullotrophic hormone," as well as parenteral administration of purified somatotrophic hormone(3), can stimulate growth of the chromaffin-cells in adrenals of laboratory rodents. However, none of these changes can be regularly reproduced and definite proof is lacking that any pituitary-preparation exerts a specific effect upon the function or structure of medullary cells. The generally recognized adrenotrophic hormone of the pituitary is called "adrenocorticotrophic" precisely because of its selective effect upon the cortex. We have long been searching for possible medullotrophic actions of ACTH-preparations. We were never able to demonstrate any such effects however, excepting the dilatation of the medullary blood-vessels and a suggestion of hypertrophy in the chromaffin-cells, which were thought to be merely secondary results of the increased blood-flow and the consequent improvement in the nutritional status of the gland as a whole. On the other hand, recently, using the PAS-technic(4), we quite regularly observed definite changes in the adrenal medulla of rats treated for various purposes with certain "long-acting" ACTH-preparations.(5). To illustrate the histologic characteristics of these changes it will suffice to present one such experiment here, since the results were uniformly the same whenever 20 I.U. per day or more was given to rats during 2 weeks or more.

Experimental. In a typical experiment, 20 I.U. of "Duracton" (Nordic Biochemicals Ltd., Montreal) was given subcutaneously, twice daily, to 10 female Sprague-Dawley rats having an average initial body-weight of 100 g. This ACTH-preparation contains 24 I.U./mg, and is made available with carboxymethylcellulose (added as a retarding agent) in an aqueous solution containing 20 I.U./ml. This treatment inhibited growth, so that the treated animals gained only an average of 14 g, while a group of 10 normal control rats gained an average of 42 g during the period of observation. All animals were killed after 14 days, at which time the mean adrenal-weight was 42 mg (range 37-47) in the controls and 92.5 mg (range 90-104) in the ACTH-treated animals. Immediately after autopsy the adrenals were fixed in susa-mixture, embedded in paraffin and stained with hematoxylin-eosin.

The slides again gave the impression that only the cortex participates in the pronounced adrenal enlargement. The individual medullary cells were not conspicuously hyperplastic, although the medullary vessels were markedly dilated. However, slides subsequently stained with the PAS-method revealed numerous rust-colored darker cell-elements throughout the medulla. Some of these cells are elongated or roundish, while others have a typical epithelioid appearance and resemble the adjacent chromaffin-cells. Occasional rust-colored granules were also seen in the cortical cells, especially in the ectopic cell-nests within the medulla, as well as in the cells of the glomerulosa and reticularis layers. Some of the reticulo-endothelial cells, which line the cortical sinusoids, likewise contain such granules (Fig. 1).

The general appearance of many among these cells is reminiscent of mast-cells. We have therefore stained additional sections of these adrenals with such selective mast-cell dyes as neutral red, acridine yellow, toluidine blue and Unna's mixture. The many granules in the mastocytoid cells of the medulla, as well as the individual granules within the cortical cells, stained uniformly, like mast-

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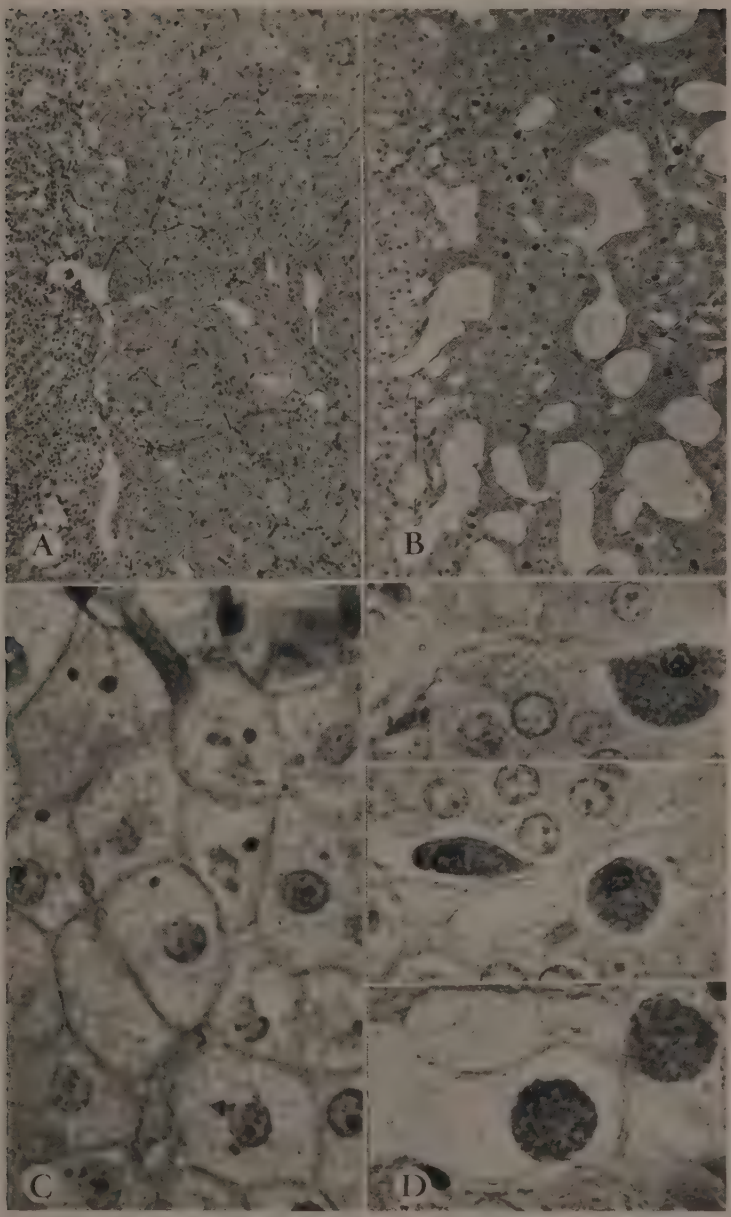

FIG. 1. A. Inner cortex (left) and medulla, under low magnification, in an untreated control rat. B. Same region in an ACTH ("Duracton") treated rat. Black mastocytoid elements are distributed throughout the chromaffin-tissue of the medulla. The medullary veins are greatly dilated. C. High magnification of a cell-region from the fasciculata of an ACTH ("Duracton") treated rat. Note individual small black granules (which stain like mast-cell granules) in the cortical cells. Their size is usually approximately that of a nucleolus and only a few such granules tend to appear within any one cortical cell. D. Three fields from the medulla shown in "B." Note various types of mastocytoid elements, some of which are epithelioid, some spindle-shaped and some rounded. All the sections in this plate were stained with the PAS-stain, which lends itself particularly well for black-and-white photography.

cells (selectively, but orthochromatically, with neutral red and acridine yellow, metachromatically with Unna's mixture and toluidine blue). Some material with similar staining properties was also found in the blood of the adrenal venules; here it usually adhered to erythrocytes.

A comparison with typical mast-cells was then made by staining sections of the thymus of the same animals on the same slide with their adrenals. It was found that the ordinary mast-cells of the connective tissue in the thymus are smaller, their shape is more regularly ovoid and they contain much smaller and more intensely staining granules than the medullary mastocytoid elements.

Discussion. On the basis of the evidence available to us, it is impossible to advance any hypothesis concerning the possible histogenesis or function of the mastocytoid cells. It should be pointed out however that, after treatment with ACTH-preparations without added carboxymethylcellulose, we have not observed any such mastocytoid elements in the medulla, nor metachromatic granulations in the cortical cells. On the other hand, control experiments with the carboxymethylcellulose-containing solvent alone, gave positive results. Presumably, the mastocytoid elements in the adrenal medulla are due to ingestion by histiocytes of metachromatically staining carboxymethylcellulose granules.

Summary. Observations on rats indicate that treatment with large doses of an ACTH-preparation, to which carboxymethylcellulose was added as a retarding agent, results in the development of mast-cell-like elements within the adrenal medulla. At the same time, in some cortical cells and in adrenal-vein blood, occasional granules appear, which give the characteristic staining reactions of mast-cell granules. Carboxymethylcellulose - solutions containing no ACTH produce similar changes.

1. Anselmino, K. J., Herold, I., and Hoffman, F., *Arch. f. Gynakologie*, 1934, v158, 531.

2. Collip, J. B., *Am. J. Obstetr. and Gyn.*, 1940, v39, 187.

3. Moon, H. D., Simpson, M. E., Li. C. H., and Evans, H. M., *Cancer Res.*, 1950, v10, 364.

4. McManus, J. F., *Am. J. Path.*, 1948, v24, 643.

5. Selye, H., in: Selye, H., and Heuser, G., *Fifth Annual Report on Stress*, 1955, M. D. Publ., New York.

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Further Observations on L Forms of *Alpha*-hemolytic Streptococci.*† (22133)

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Isolation of L-type cultures from 2 strains of *alpha*-hemolytic streptococci was described in 1953(1). Many attempts at isolation of these forms from *alpha*-hemolytic streptococci in previous years had been unsuccessful. This paper contains the description of two methods which permit with some regularity the finding of strains of *alpha*-hemolytic streptococci which produce L forms. Data are also presented concerning the occurrence of such strains in humans and observations on a pleomorphic strain of *alpha*-hemolytic streptococcus which produced L forms spontaneously without the influence of penicillin.

After the characteristic appearance of L colonies of *alpha*-hemolytic streptococci and the conditions under which they grow became

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known, specimens from the human mouth, urethra and vagina were cultivated on appropriate plates. Bacterial growth was inhibited in a part of the plate by penicillin. In cultures made from the mouth, colonies of pleuropneumonia-like organisms usually developed on the inhibited areas and among these occasionally were found much larger colonies which corresponded in appearance to L-type colonies of streptococci. In 3 out of approximately 80 cultures examined, it was possible to subculture these large L-type colonies. They were first subcultured on penicillin plates. When the cultures were allowed to age in the absence of penicillin, *alpha*-hemolytic streptococci appeared in all. Each of these streptococcus cultures produced L-type growth on exposure to penicillin and evidently originated from the reversion of the L forms. The development of L-type colonies corresponding to those of streptococci was not observed in cultures made from the urethra or vagina although streptococcus colonies often developed abundantly on the plates.

These observations were made on the usual horse serum medium containing 0.5% NaCl. After it was observed that on plates containing a high concentration of NaCl or some other electrolytes many *beta*-hemolytic streptococci produced L forms(2,3) it seemed of interest to examine the behavior of *alpha*-hemolytic streptococci on such media.

Preliminary experiments were made by planting tooth scrapings and throat swabs on horse serum agar containing 3% NaCl and exposing part of each plate to penicillin. The development of L colonies of streptococci was observed only rarely on these plates. In a more systematic study made with throat swabs, a mixture of many colonies of *alpha*-hemolytic streptococci from a blood agar plate was inoculated on another plate to check the purity of the culture. From this culture large inocula were transferred to broth and the test plates were then heavily seeded with this broth culture. In this way each test plate received an inoculum originating from a number of colonies found in the same specimen. This procedure was adopted because trials with the descendants of single colonies had remained negative. An increased NaCl content of the media only rarely favored the development of L-type colonies. However, an increase in the phosphate content either of the sodium or potassium salt, had a marked effect. Abundant development of L-type colonies was observed with concentrations of phosphate between 0.2 and 0.4 M. Only one strain gave a slight growth of L forms with 0.1 M. Concentrations higher than 0.4 M were not tested. Ten out of 40 of such mixed cultures produced L forms on the phosphate plates. Two of the 40 produced L-type colonies with high concentrations of NaCl also, but less abundantly than with the phosphate. The influence of the phosphates was not due to differences in pH as the pH was the same in the chloride and phosphate plates. None of these 40 cultures produced L-type colonies without an increased salt concentration.

Subculture of these L forms was successful only on plates with an increased phosphate content indicating that the high concentration of phosphate ions was important for their growth. The transformation of the cocci into the L forms was probably induced by the penicillin, just as on the regular media. In the case of the strains of *alpha*-hemolytic streptococci which produced L-type colonies on the usual horse serum media, a high phosphate concentration in the medium neither enhanced the transformation of such strains into L forms nor was necessary for the subculture of the L forms.

On the basis of these observations the following procedure can be recommended to obtain L-type cultures of *alpha*-hemolytic streptococci. Plates are made using a tryptic digest of beef heart or beef heart infusion as base, 1.3% agar, and 10% horse serum inactivated at 56°C for 30 minutes. Three ml of a mixture of 20% Na_2HPO_4 and 1.4% KH_2PO_4 is added to 15 ml of the medium. The plates are heavily inoculated as described above. The plates are incubated for 2 hours to allow multiplication of the cocci and then about 1000 units of penicillin are deposited in a small trough cut in the agar. The plates are incubated at 36°C for 4 to 5 days under anaerobic conditions using the Fortner biological method. The behavior of the cultures was consistent. Six strains which originally

produced L forms continued to produce such forms in 4 to 13 subcultures when the transfers were made with heavy inocula. Three to five subcultures of seven strains, which originally did not produce L forms, did not yield this type of growth. The use of an agar base of different composition did not increase the percentage of positive strains.

The observations described indicate that approximately one out of 4 humans harbors in his mouth *alpha*-hemolytic streptococci which, under the conditions employed, produce L-type colonies. Further study has shown that only a few of the single colonies in the throat cultures of such persons will produce L colonies. One throat culture known to be positive was studied more thoroughly and only the descendants of 1 out of 20 single colonies gave positive results. Further subcultures of these 20 colonies behaved similarly. The descendants of negative colonies were always negative. Subcultures obtained by transferring large inocula from a positive strain were always positive. However, only about one-fifth of the descendants of isolated colonies remained positive. This was followed through 4 generations. These observations indicate that not all but only a certain percentage of the cocci in a positive colony transmit to their descendants the ability to produce L forms under the conditions of these experiments. A similar observation was made previously in this laboratory with a Gram positive bacillus(4).

An interesting strain of *alpha*-hemolytic streptococcus was isolated on several occasions from the blood of a patient in the weeks preceding his death.§ The source of the septicemia was an old kidney abscess; no endocardial lesions were found at autopsy. The organisms were extremely pleomorphic both in the original broth flasks and in transplants on blood agar plates without penicillin. Normal cocci were not observed and the cultures consisted of large round bodies, small granules and long filaments, some with swellings. The cultures resembled those of *Streptobacillus moniliformis*. The nature of the organisms was established only after several transfers, when the usual coccal form of growth was resumed. When transferred immediately after isolation to horse blood and horse serum agar plates without penicillin and without increased electrolyte concentration, L colonies developed spontaneously among the pleomorphic coccus colonies. When the cultures were exposed to penicillin only L colonies developed.

Discussion. The L forms of bacteria can be recognized at present only by the growth of characteristic colonies. This criterion was used in all cases reported in this paper. The occurrence of large bodies or granules in the cultures, as accepted by several authors,‖ is not sufficient proof of transformation of bacteria into L forms. Such morphological elements are often present in cultures without signs of multiplication. The expressions "L forms," "L-type growth" and "L cultures" are synonymous and refer to the same fundamental phenomena.

The L-type cultures described in this report undoubtedly originated from *alpha*-hemolytic streptococci. The macroscopical and microscopical appearance of the cultures is characteristic, and differs from that of PPLO which could conceivably contaminate the cultures. The serological specificity of the L forms studied earlier and in the course of the present studies was similar to that of the parent streptococci. The observation that streptococci reappeared in the cultures of L forms and that these cocci reproduced L forms under appropriate conditions is further evidence for the relation between the cocci and these L forms.

The tendency of strains of *alpha*-hemolytic streptococci to produce L forms is as variable as in the other species. The behavior of these strains, as far as the production of L forms is concerned, is in many respects similar to that observed in *Bacteroides*(5). One strain of *alpha*-hemolytic streptococcus produced L forms spontaneously on the usual media. A few strains produced L forms on similar

§ We are indebted to Lawrence J. Kunz, bacteriologist of the Massachusetts General Hospital, for this strain and many others mentioned in this publication.

‖ See discussion in (3).

media under the influence of penicillin. High concentrations of NaCl increased slightly and high concentrations of phosphate increased markedly the number of strains which produced L forms. The behavior of the descendants of individual coccal chains of a strain which produced L forms was variable. In some strains the majority of the cocci present in a single colony lost the ability to produce L forms.

It is of interest that in several species such as *Bacteroides* and the hemophylic bacteria, which are the usual inhabitants of the human body, pleomorphism and spontaneous production of L forms is more frequent among the strains isolated from pathological processes than among those isolated from their usual habitat. Similarly, the strain of *alpha*-hemolytic streptococcus which produced L forms spontaneously originated from an abscess of long duration, and was characterized by extreme pleomorphism. This relation cannot be due entirely to the influence of antibiotics, because some of these observations were made before they came into use.

Summary. High concentrations of potassium and sodium phosphates incorporated in the media markedly increased the number of strains of *alpha*-hemolytic streptococci which produced L-type growth when exposed to penicillin. High concentrations of NaCl produced this effect only slightly. Using the phosphate media, strains of *alpha*-hemolytic streptococci which produced L forms were found in about one-fourth of the throat cultures examined. One unusual strain of *alpha*-hemolytic streptococcus produced L forms spontaneously without exposure to penicillin.

1. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 579.
2. Sharp, J. T., *ibid.*, 1954, v87, 94.
3. Dienes, L., and Sharp, J. T., *J. Bact.*, in press.
4. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 30.
5. ———, *J. Bact.*, 1948, v56, 445.

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Endogenous Formation of Hippuric Acid.* (22134)

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(Introduced by Emil L. Smith.)

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Occurrence of hippuric acid in mammalian urine has been known since its isolation by Liebig(1) in 1829. It has usually been supposed to arise by conjugation of benzoic acid of dietary origin with glycine in the body, and a great part of biochemical investigations of hippuric acid formation have been concerned with glycine metabolism. Recently, presence of hippuric acid in urine of fasting humans was reported and a possible endogenous origin was suggested for it(2). Shortly thereafter, Schreier *et al.*(3) observed a dilution in specific activity of radioactive benzoic acid in fasting rats, and suggested that an endogenous formation of benzoic acid could explain their results. In addition, they cited other observations, unsupported by experimental data, which were in agreement with this idea. Our investigation indicates that in humans and in the white rat there is a small amount of hippuric acid produced endogenously and that in the rat the aromatic ring of phenylalanine can, to a slight extent, serve as a precursor for the benzoic acid moiety. A major proportion of hippuric acid present in urine of humans and of rats receiving a natural diet is, however, derived from dietary precursors.

Methods. Albino rats (Sprague-Dawley), weighing 300 to 400 g, were fed either Purina

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chow or lactalbumin-dextrin diet which has been described earlier(4). Each animal was allowed 15 g of food daily and given water *ad libitum;* fasting animals were allowed water. For experiments in which a laboratory diet was used with humans, the basal diet contained lactalbumin, sucrose, a vegetable oil (Wesson oil), vitamins and minerals(5). The amounts allowed in g/kg/day were lactalbumin, 1; sucrose, 0.8; fat, 0.8; mineral salts (No. 2, General Biochemicals, Inc.), 0.2, and vitamins. Rat urine was collected with toluene as a preservative; 24-hour samples were acidified to pH 2, extracted twice with 3 volumes of ethyl acetate, and the ethyl acetate was concentrated *in vacuo*. Final volume was adjusted to contain about 0.2 mg of hippuric acid/ml of ethyl acetate. Human urine was collected and frozen immediately; 24-hour samples were assayed for creatinine and organic acids of an aliquot were extracted by the standard procedure reported previously(6). Hippuric acid concentration of the ethyl acetate extracts was estimated by modification of procedure described by Gaffney *et al.*(7). Aliquots which contained between 2 and 10 μg of hippuric acid were placed on Whatman No. 1 filter paper along with 1 to 10 μg amounts of authentic hippuric acid, and the chromatograms developed with isopropyl alcohol-aqu. ammonia-H_2O (8:1:1) for 16 hours at 25°C. Hippuric acid (R_f = 0.59) was visualized by spraying with *p*-dimethylaminobenzaldehyde/Ac_2O/NaOAc reagent(4) followed by either heating at 100°C for 5 minutes or letting the sprayed chromatograms stand overnight; the latter procedure results in a better background on chromatograms. The amount of hippuric acid was estimated by comparing intensity of color of unknowns and standards as seen with a Kodak cold light X-ray illuminator. Recovery experiments showed that the procedure for rat urine gave a quantitative recovery, and that procedure used for human urine gave approximately 90% recovery of hippuric acid. Accuracy of estimation is within ±20%, which is adequate for purposes of these experiments.

The *results* (Table I) show that a major portion of hippuric acid excreted by rats receiving a chow diet arises from some precursor in the diet; no preformed hippuric acid could be detected in the chow. When animals were given lactalbumin diet, hippuric acid excretion fell during the first few days to 3 to 4 mg/kg/day and then remained constant. Fasting or inclusion of 2% sulfasuxidine in the diet to modify intestinal microorganisms did not lead to significant decrease in hippuric acid excretion nor did oral administration or intraperitoneal injection of phenylalanine lead to increased excretion.

TABLE I. Excretion of Hippuric Acid by the Rat.

Regimen	No. of animals	Days studied	Avg daily hippuric acid excreted (mg/kg body wt/day)
Laboratory chow	6	4	22.5 ± 6.1
Laboratory diet			
Day 1	6	1	14.0 ± 5.0
2	6	1	8.8 ± 4.0
3	6	1	5.6 ± 2.5
4	6	1	5.0 ± 1.0
5	3	1	4.3 ± .3
6	3	1	3.9 ± .6
Fasting			
Day 7	3	1	4.4 ± 1.4
8	3	1	3.6 ± .2
9	3	1	3.5 ± .1
10	3	1	3.6 ± .5
11	3	1	3.6 ± .4
12	3	1	3.6 ± .3
13	3	1	3.4 ± .3
Synthetic diet + 2% sulfasuxidine—5th and 6th days	3	2	3.1 ± .3
Synthetic diet + 2% sulfasuxidine + 200 mg L-phenylalanine intraper.	3	3	3.3 ± .2

The results obtained with humans (Table II) were similar to those with rats. A much higher excretion of hippuric acid was observed in individuals eating a natural diet than in the same persons after they had eaten a synthetic diet similar to that fed the rats. Excretion of hippuric acid decreased rapidly as soon as synthetic diet was commenced, and then remained relatively constant, with different individuals excreting different amounts, ranging from 1 to about 3 mg/kg body weight/day—values similar to those observed with rats.

Some caution is mandatory when a color reaction is applied to the analysis of materials

TABLE II. Hippuric Acid Excretion in the Human Receiving a Laboratory Diet.

	Hippuric acid excretion (mg/kg/day)					
	Natural diet	Consecutive days of laboratory diet				
		1	2	3	4	5
MA (♂)	14.3	4.4	.7	.5	.7	.7
VP (♂)	7.1	2.3	1.6	1.3	1.5	1.7
PW (♀)	58.0	23.0	3.3	2.4	2.4	2.0

of biological origin. To assure that the material estimated with the reagent of Gaffney *et al.*(7) actually was hippuric acid, the final 24-hour urine sample of one person receiving the synthetic diet for 5 days was extracted in the usual manner, and hippuric acid was isolated from the ethyl acetate extract (200 ml, containing an estimated 100 mg of hippuric acid) with the use of a modified counter-current distribution procedure. Thirty-eight mg of impure hippuric acid, obtained in the first crop, was recrystallized (Norit) from 0.6 ml of hot water to yield 16 mg of white material. The isolated compound was identical with authentic hippuric acid in all its physical properties. The demonstration that hippuric acid actually could be isolated from urine of an individual receiving a well-defined diet supports the conclusion that the material estimated as hippuric acid in this work actually was hippuric acid and not another chromogen with similar chromatographic behavior.

Preparation of 3-C^{14}-L-phenylalanine. 77.9 mg of 3-C^{14}-DL-phenylalanine hydrochloride (Tracerlab, Inc., 3.84 Mc/mg) and 227.1 mg of inert DL-phenylalanine were mixed to provide 291.0 mg of DL-phenylalanine (1 Mc/mg). This material was resolved by modification of the method of Wretlind(8). 115 mg of L-phenylalanine, $\alpha]_D^{24} = -34°$ (c,l,H_2O) (−35°)(8), and 101 mg of D-phenylalanine, $\alpha]_D^{24} = +33°$ (c,l,H_2O) were obtained.

Isolation of labelled hippuric acid from rat urine. Hippuric acid was extracted from 24-hour samples of urine with ethyl acetate as described above, and a small aliquot used for estimating total quantity present. Ethyl acetate solution was then extracted 4 times with small portions of saturated $NaHCO_3$ solution and 30 mg of carrier hippuric acid was added to the combined bicarbonate extracts. The solution was cooled to 0°, acidified to pH 2 by the addition of 6 N HCl, and allowed to stand overnight in a refrigerator. The hippuric acid was recrystallized from hot water. Hippuric acid, obtained by the above procedure, was hydrolyzed by heating with 2 ml of 6 N HCl at 110° in a sealed tube for 12 hours. The acidic solution was extracted 3 times with 5 ml portions of ether, the combined ether extracts were evaporated to dryness, and benzoic acid was purified by sublimation at 120°; m.p., 122°. For counting, aliquots of urine, of the ethyl acetate extract, and ethyl acetate solutions of hippuric and benzoic acids were placed on aluminum discs (1-inch diameter). Radioactivity was measured on duplicate samples with an internal gas flow counter having a geometry of about 50%. Sufficient counts were measured to allow a counting error of ±5% and the counts were corrected for background and self-absorption. The results of tracer experiments with 3-C^{14}-L-phenylalanine and uniformly labelled C^{14}-glucose are given in Table III.

Discussion. The experiments reported here serve to establish 2 points: first, there is a small endogenous production of benzoic acid in rat and human, and second, this benzoic acid probably is formed in some indirect and, as yet unknown, manner from phenylalanine.

By far the greater proportion of the hippuric acid excreted by rats and humans who are ingesting a natural diet has its origin in dietary precursors—either combined benzoic acid present in plant materials or, in the case of humans, additional small amounts of sodium benzoate, which is added to some foods as a preservative. After the institution of well-defined laboratory diet, amount of hippuric acid excreted in urine of both species decreased rapidly over 2 to 3 days to a fraction of the amount observed originally. The amount, on a body weight basis, of this endogenous hippuric acid excreted was comparable in human and rat.

In the earlier report of presence of hippuric acid in urine of a fasting human(2), the amount of hippuric acid excreted was somewhat greater than that observed here. This may have resulted because of too short a

TABLE III. Results of Tracer Experiments with Rats.

Compound administered by intraper. inj. ⟶	C^{14}-Glucose; one portion		3-C^{14}-L-Phenylalanine; 3 portions at 6 hr intervals		3-C^{14}-L-Phenylalanine; one portion		
	Fasting		Laboratory diet		Fasting		
Days studied ⟶	2nd	3rd	6th	7th	2nd	3rd	4th
Amt of compound given (mg)	65.5		1.		1.		
C^{14} given (cts/min. $\times 10^{-6}$)	.95		.90		1.		
C^{14} in 24 hr urine (cts/min.)	7530	990	4610	1760	8790	4370	3000
Proportion of C^{14} excreted in urine (%)	.793	.103	.512	.195	.879	.437	.30
C^{14} in EtOAc extract (cts/min.)	870	380	1760	—	7970	3330	2980
Proportion of urinary C^{14} in EtOAc (%)	11.5	38.5	38.2	—	90.6	76.2	99.4
Hippuric acid excreted (mg)	1.0	1.	1.	1.	1.	1.	1.
Hippuric acid isolated (30 mg carrier added) (mg)	22.	—	23.7	—	24.	—	—
Sp. act. of isolated hippuric acid (cts/min./mg)	0	—	17.5	6.9	20.1	9.5	7.
Sp. act. of benzoic acid from isolated hippuric acid (cts/min./mg)	—	—	14.8	—	17.4	—	—
C^{14} in benzoic acid portion of hippuric acid (cts/min.)	0	—	459	—	540	—	—
Proportion of C^{14} excreted in benzoic acid moiety of hippuric acid (%)	0	—	0.051	—	0.054	—	—

period of fasting before urine was collected; the data of Table I show that a constant low excretion of hippuric acid was not observed until the third day of ingestion of laboratory diet. Presumably, this length of time is required for material of dietary origin present in intestinal tract to be cleared from the body. A similar finding was made in connection with excretion of *m*-hydroxy-hippuric acid, which is also derived in large part from some dietary precursor(6).

The tracer experiments with C^{14}-labelled phenylalanine, designed to test the possibility that benzoic aicd might arise directly from phenylalanine by removal of the 2 terminal carbon atoms of the side chain, clearly showed that, although phenylalanine may definitely serve as precursor to a very limited extent, it can hardly be a direct precursor. The very small amount of radioactivity of administered 3-C^{14}-phenylalanine appearing in hippuric acid excreted was present in the benzoic acid portion of the hippuric acid rather than in the glycine portion, in which it might have occurred as the result of glycine formation from acetoacetate or acetate formed by complete breakdown of phenylalanine. The possibility that *de novo* synthesis of a small amount of C^{14}-containing benzoic acid from acetate might have been carried out by intestinal microorganisms was considered and was tested by administration of uniformly labelled C^{14}-glucose, which has been shown to serve as precursor for microbial synthesis of aromatic rings. Hippuric acid excreted after administration of labelled glucose did not contain a detectable amount of C^{14}.

Thus, the observation of a significant production of endogenous benzoic acid in the rat, which was indicated by the dilution of administered C^{14}-benzoic acid in the work of Schreier *et al.*(3), has been confirmed in the present experiments. In addition, it has been shown that phenylalanine serves but poorly as precursor, and is probably first converted to some unknown intermediate which gives rise to benzoic acid. Utilization of 3-C^{14} rather than ring-labelled phenylalanine leaves open the possibility that phenylalanine might have served as precursor for C^{14}-benzoic acid,

which then underwent decarboxylation and recarboxylation, losing the label in the process. In addition to absence of known metabolic transformations of this type, however, lack of incorporation of C^{14} from C^{14}-labelled glucose into the hippuric acid renders this possibility unlikely.

Summary. 1. Humans and rats receiving a purified laboratory diet excrete hippuric acid, 1 to 3 mg/kg/day. This amount appears to be formed endogenously and its excretion in the rat is not affected by fasting, by inclusion of sulfasuxidine in the diet, or by injection of phenylalanine. 2. 0.05% of the radioactivity of 3-C^{14}-L-phenylalanine injected into a rat was excreted in 24 hours in the form of hippuric acid. Further experiments suggested that endogenous hippuric acid could not be derived from phenylalanine in any direct manner.

1. *Beilsteins Handbuch des organischen Chemie,* 1926, v9, 225, J. Springer, Berlin.

2. Stein, W. H., Paladini, A. C., Hirs, C. W. H, and Moore, S., *J. Am. Chem. Soc.,* 1954, v76, 2848.

3. Schreier, K., Altman, K. I., and Hempelmann, L. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 61.

4. Shaw, K. N. F., and Armstrong, M. D., *ibid.,* 1955, v88, 673.

5. Armstrong, M. D., Wall, P. E., and Parker, V. J., *J. Biol. Chem.,* 1956, v218, 921.

6. Armstrong, M. D., Shaw, K. N. F., and Wall, P. E., *ibid.,* 1956, v218, 293.

7. Gaffney, G. W., Schreier, K., DiFerrante, N., and Altman, K. I., *ibid.,* 1954, v206, 295.

8. Wretlind, K. A. J., *ibid.,* 1950, v186, 221.

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Studies on Thrombin Formation. (22135)

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Treatment of plasma with barium sulfate has been reported to remove the following clotting factors: Prothrombin, the serum prothrombin accelerator system*(1), plasma thromboplastin component*(2) and plasma thromboplastin*(3). However, antihemophilic factor* (AHF) and plasma-accelerator-globulin* (PacG) are supposedly not removed by the adsorbent(4). The present study is concerned with the influence of AHF and of PAcG on the rate of thrombin formation in a reaction mixture containing calcium ions and platelets as well as factors removed from bovine plasma by barium sulfate (plasma eluate). Concomitant investigations were made on the influence of a barium sulfate eluate from rabbit serum (serum eluate) on the rate of thrombin formation.

Materials. Bovine plasma: Oxalated bovine blood (9 parts of blood and 1 part of 3.7% potassium oxalate in 1% oxalic acid) was collected at the slaughter house and centrifuged at 4°C. *Veronal buffer*: A stock solution was made by dissolving 19.428 g sodium acetate • $3H_2O$ and 29.428 g sodium diethylbarbiturate in one l of distilled water. Veronal buffer of pH 7.2 was used throughout and consisted of 100 ml of the stock solution, 40 ml of 8.5% sodium chloride, 100 ml of 0.1 M hydrochloric acid and 260 ml of distilled water. *Bovine platelets* were obtained from oxalated bovine plasma by differential centrifugation according to the procedure described by Seegers and his associates(5). After the final washing with 0.9% NaCl, the platelets were dried with acetone and then pulverized in a mortar. Suspensions of the dried platelet material were made in veronal buffer. *Bovine plasma eluate*: Oxalated bovine plasma was stirred at room temperature for 10 minutes with $BaSO_4$ (50 mg/ml), centrifuged at 4°C and the supernatant plasma decanted. The $BaSO_4$ was washed successively with 0.9% NaCl, 0.1 M sodium oxal-

* For a different terminology of these factors reference is made to Appendix, The Coagulation of Blood. Method of Study. Edited by L. M. Tocantins, Grune and Stratton, N. Y. City, 1955.

ate, and 0.9% NaCl, the volumes of each washing being equal to the original plasma volume. The $BaSO_4$ was then eluted twice with 0.1 M sodium citrate solution (1/10 of the original plasma volume) by stirring for 10 minutes at room temperature, and then centrifuging and decanting. The two citrate eluates were combined and dialyzed against distilled water at 4°C until free of citrate and then lyophilized. A given amount of the dried preparation was dissolved in veronal buffer, corresponding to definite plasma concentrations. *Rabbit serum eluate* was prepared from stored aged rabbit serum of low prothrombin content according to the procedure described for the plasma eluate. An amount of the dried material was extracted with veronal buffer equivalent to serum concentration, centrifuged for 20 minutes at 4°C and the clear supernatant was employed in the various tests. *Plasma accelerator globulin (PAcG)* was prepared according to the method of Lewis and Ware(6) from bovine oxalated plasma after treatment with $BaSO_4$. The final preparation was dissolved in a volume of 0.9% NaCl, corresponding approximately to 1/5 of the original plasma volume, and kept frozen in small aliquots until used. *Antihemophilic globulin factor (AHF)* was prepared according to the procedure described by Brinkhous and his associates(7,8). $BaSO_4$-treated oxalated bovine plasma was heated for 10 minutes at 52°C to remove fibrinogen. After centrifugation, ammonium sulfate was added to the supernatant to 25% saturation. After one hour at 4°C, the resulting precipitate was removed by centrifugation, dissolved in distilled water and dialyzed at 4°C against 0.9% NaCl. Final volume of the AHF solution was adjusted to the original plasma volume, and kept frozen in small aliquots until used. *Fibrinogen* was prepared from oxalated bovine plasma according to the method of Seegers and his associates(9). Before dilution, the fibrinogen solution was treated with $BaSO_4$ (100 mg/ml) to remove any absorbable clotting factors which may have been present. For the test, the fibrinogen was diluted with 0.9% NaCl to a concentration of 0.4%. The fibrinogen preparation contained 89% clottable protein.

Methods. A. One-stage thrombin formation procedure: The total incubation mixture consisted of 1.25 ml plasma eluate (2 times plasma concentration) in veronal buffer, 0.6 ml AHF solution, 0.6 ml PAcG solution, 0.55 ml serum eluate (serum concentration) in veronal buffer, 0.15 ml platelets suspension (1.5 mg/ml veronal buffer), and 0.3 ml $CaCl_2$ (0.125 M). Veronal buffer was substituted for one or more of the above reagents in the various experiments. On the addition of the $CaCl_2$ to the other factors and agitation of the mixture, a stop watch was started and the reaction mixture was incubated at 26°C. Thrombin formation was determined at timed intervals by the addition of 0.1 ml of the reaction mixture to a fibrinogen solution (0.3 ml veronal buffer and 0.1 ml fibrinogen) at 37°C. When the clotting times (as obtained by the tilt method) were below 12 seconds, the 0.1 ml portions of the reaction mixture were suitably diluted with veronal buffer before addition to the fibrinogen solution. The recorded clotting times were interpreted in terms of National Institutes of Health (NIH) thrombin units from a curve prepared with a standard NIH thrombin preparation.† *B. Two-stage thrombin formation procedure*: In

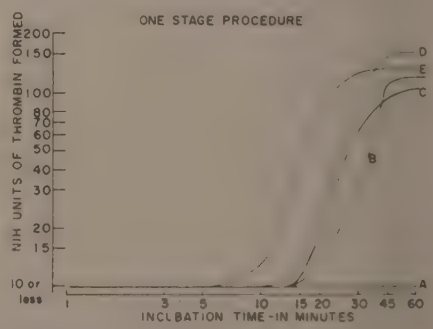

FIG. 1. Formation of thrombin in plasma eluate, platelets and $CaCl_2$ in the presence of serum eluate, AHF, PAcG and AHF plus PAcG. A, plasma eluate, platelets and $CaCl_2$; B, plasma eluate, platelets, $CaCl_2$, and serum eluate; C, plasma eluate, platelets, $CaCl_2$ and AHF; D, plasma eluate, platelets, $CaCl_2$ and PAcG; E, plasma eluate, platelets, $CaCl_2$, AHF and PAcG.

† Kindly supplied by Biologics Control Laboratory, National Institutes of Health, Bethesda, Md.

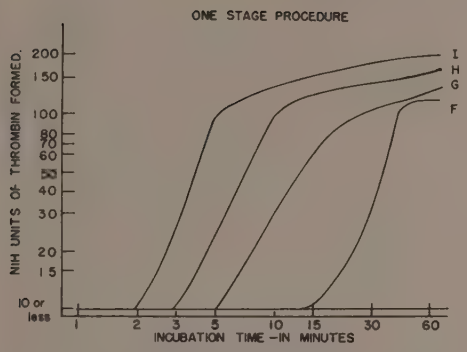

FIG. 2. Formation of thrombin in plasma eluate, serum eluate, platelets and $CaCl_2$ in the presence of PAcG, AHF and PAcG plus AHF. F, plasma eluate, serum eluate, platelets and $CaCl_2$; G, plasma eluate, serum eluate, platelets, $CaCl_2$ and PAcG; H, plasma eluate, serum eluate, platelets, $CaCl_2$ and AHF; I, plasma eluate, serum eluate, platelets, $CaCl_2$, AHF and PAcG.

this procedure serum eluate, platelets and $CaCl_2$ with and without AHF or PAcG were incubated for 20 minutes at 26°C and the plasma eluate was then added to the incubation mixture. The volumes for the different reagents in this procedure were the same as in the one-stage thrombin activation procedure. The prothrombin converting activity of the incubation mixture was assessed by recording the ability and the velocity of the reaction mixture to form thrombin upon addition of a plasma eluate and incubation at 26°C. The rate of thrombin formation was followed in the same manner as in the one-stage thrombin formation procedure. Details of representative individual experiments are included in Fig. 1, 2 and 3.

Results. In any interpretation of the various reaction rates of the different factors controlling the formation of thrombin, consideration must be given to the law of mass action. Cognizance must also be taken of the possible presence of other clotting factors or inhibitors as impurities in the preparations employed, however small these contaminations may be. Nevertheless, it is felt that only quantitative and not qualitative differences may occur when corresponding reactions are executed with more purified preparations. Data presented in the various figures constitute mean values of 3 or 4 identical experiments. The clotting times did not differ by more than 10% for each group of identical experiments.

In the curves of Fig. 1 and 2, depicting simultaneous mixing of the reactants, the rate of thrombin formation represents the summation of all intermediate interactions involved in the elaboration of thrombin. As can be seen from curve A of Fig. 1, incubation of the bovine plasma eluate with platelets and $CaCl_2$ yielded only traces of thrombin. However, in the presence of either serum eluate (curve B of Fig. 1), or AHF (curve C of Fig. 1), or PAcG (curve D of Fig. 1), prothrombin conversion became more pronounced. As can be seen from Fig. 1 marked thrombin formation did not commence until after 10 to 15 minutes of incubation. Once thrombin formation began, the rate of conversion of prothrombin to thrombin increased rapidly. In the presence of both AHF and PAcG (curve E of Fig. 1), thrombin evolution was relatively fast: 80 NIH units were elaborated after 20 minutes compared with 29 units with PAcG, 20 NIH units with AHF and 15 NIH units with serum eluate. Addition of either serum eluate, or of PAcG, or of AHF, or of AHF and PAcG seemed to influence mainly the rate but not the amount of thrombin formation.

The data in Fig. 2 indicate that addition of

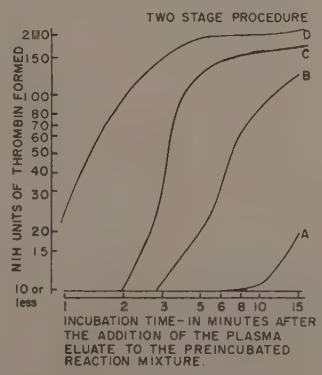

FIG. 3. Formation of prothrombin converting activity on 20 min. incubation of serum eluate, platelets and $CaCl_2$ in the presence of PAcG, AHF and PAcG plus AHF. A, serum eluate, platelets and $CaCl_2$; B, serum eluate, platelets, $CaCl_2$ and PAcG; C, serum eluate, platelets, $CaCl_2$ and AHF; D, serum eluate, platelets, $CaCl_2$, PAcG and AHF.

serum eluate to the plasma eluate enhanced the accelerating influence of either PAcG or AHF in the utilization of prothrombin. In the presence of AHF (curve H of Fig. 2) there was both an increase in the yield and the rate of thrombin formation after 10 minutes of incubation (112 NIH units) compared with PAcG (30 NIH units, curve G of Fig. 2). These observations substantiate the concept of Brinkhous and his associates(4) that AHF augments the utilization of prothrombin. In the presence of both AHF and PAcG (curve I of Fig. 2), the lag period of prothrombin conversion was further shortened; about 26 NIH thrombin units were formed within 3 minutes, while only traces of thrombin were elaborated during the same period of incubation with either AHF or PAcG alone. Comparison of the data of Fig. 1 and 2 indicate that AHF as well as PAcG in the presence of serum eluate caused a more pronounced shortening in the prothrombin utilization than in the absence of serum eluate. The quantity of thrombin formed in a mixture of plasma eluate, platelets and calcium at any given time not only was dependent on the amount of prothrombin present but also on the rate of its conversion controlled by the presence and quantity of such factors as AHF, PAcG and those present in serum eluate. Replacing either platelets or $CaCl_2$ with veronal buffer in the experiments did not yield any measurable thrombin formation.

In an attempt to reduce further the lag period observed in the one-stage thrombin formation procedure, those factors which apparently participate in the conversion of prothrombin to thrombin were preincubated for 20 minutes at 26°C before addition of the plasma eluate. The results illustrated in Fig. 2 and 3 show that preincubation of serum eluate, platelets and $CaCl_2$ in the presence of PAcG or AHF or both PAcG and AHF before addition of the plasma eluate to the incubation mixture, resulted in a marked enhancement in the prothrombin conversion compared with the one-stage thrombin formation procedure. It appears from these data that AHF as well as PAcG interact with factor(s) in serum eluate in the presence of platelets and calcium prior to the activation of prothrombin. In the presence of AHF (curve C of Fig. 3) a more potent prothrombin activator seemed to evolve than in the presence of PAcG (curve B of Fig. 3). This is in further agreement with the finding of Brinkhous and his associates(4) that AHF greatly enhances the utilization of prothrombin. Jensen, Gray and Schaefer(10) have presented evidence that PAcG accelerates the interaction of platelets and factor(s) in a serum eluate in the presence of $CaCl_2$ yielding a prothrombin conversion factor. With both AHF and PAcG in the preincubation reaction mixture, (curve D of Fig. 3), 24 NIH thrombin units were already formed within 1 minute after addition of the plasma eluate. In the presence of AHF only (curve C of Fig. 3) and PAcG only (curve B of Fig. 3) 2.5 minutes and 5 minutes respectively were required to yield the same amount of thrombin. Replacement of platelets or of $CaCl_2$ with veronal buffer did not yield any prothrombin converting activity. Serum eluate, AHF, PAcG, platelets and $CaCl_2$ after incubation for 20 minutes at 26°C produced a clot with fibrinogen in 9 minutes, indicating the presence of only traces of prothrombin in the above preparations.

A comparison of the rates of thrombin formation (Fig. 2 and 3) shows that preincubation of AHF or PAcG or both with serum eluate, platelets and $CaCl_2$ greatly reduced the lag period in the conversion of prothrombin to thrombin. It appears from the data in Fig. 2 and 3 that regardless of whether the prothrombin converting complex was formed in one stage or two, approximately the same amount of thrombin was formed. The main difference between the one and 2-stage reaction was in the rate of prothrombin conversion. The greater velocity of thrombin formation in the 2-stage procedure indicates that during preincubation precursory interactions of certain factors had been initiated or completed.

Preincubation of either platelets, AHF and $CaCl_2$ or of platelets, PAcG and $CaCl_2$ or of platelets, AHF, PAcG and $CaCl_2$ before addition of either plasma eluate or of plasma

eluate plus serum eluate to the preincubation mixture resulted in approximately the same rates of thrombin elaboration as was found in the one-stage thrombin procedure (Fig. 1 and 2). These findings indicate that no interaction between the various factors had taken place during the preincubation period under these conditions. It appears from these observations that AHF as well as PAcG will interact with platelets and calcium ions only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity.

Discussion. The data presented point to the existence of several factors in blood which react, perhaps in stages, to produce a powerful activator of prothrombin, called the thrombokinase-complex.‡ Factors concerned in the thrombokinase forming system are AHF, PAcG, factor(s) in serum or plasma eluate, platelets and calcium ions. Apparently PAcG and AHF will react with platelets and calcium only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity. Whether the activities of the thrombokinase generated with and without AHF or PAcG differ qualitatively as well as quantitatively remains to be determined. Applying these results to the mechanism of fibrin formation in blood, the role of the different natural anticoagulants must be taken into account.

The findings reported are in agreement with the concept of Biggs(12) that the Christmas factor* (present in the eluate of plasma or serum), AHF, factor V* (PAcG), factor VII* (present in the eluate of plasma or serum), platelets and calcium ions are all required for the generation of a "complete" thromboplastin§ (thrombokinase). According to Spurling and King(13), plasma thromboplastin component‖ (present in plasma or serum eluate), AHF, platelets, rough surfaces and calcium are required for maximum thromboplastin (thrombokinase) production *in vitro*. These investigators, apparently, did not include PAcG in their concept of thrombokinase formation. Flynn and Coon(14) have presented evidence that brain thromboplastin extract, stable factor* (present in plasma or serum eluate) and labile factor* (PAcG) react together prior to the activation of prothrombin. According to Seegers(15), the plasma platelet cofactor 1* (AHF) reacts with platelet factor 3* to form threone which in combination with serum accelerator globulin* (derived from PAcG) will convert prothrombin to thrombin. Mann and Hurn (16) observed that platelets interact with a plasma—or serum factor (cothromboplastin)* before interaction with prothrombin takes place.

The data presented here as well as the observations of others already cited are in harmony with the 3 stage concept of fibrin formation as formulated by Milstone(11).

Prothrombokinase-complex ——————→ Thrombokinase complex

Prothrombin —Thrombokinase complex——→ Thrombin

Fibrinogen —Thrombin——→ Fibrin

The prothrombokinase complex would include such factors as platelets, AHF, PAcG, factor(s) in serum or plasma eluate and calcium. For the thrombokinase complex formation, platelets, factor(s) in serum or plasma eluate and calcium seem to be essential, while AHF as well as PAcG will enhance its formation. The fundamental phase of the blood clotting mechanism seems to involve the generation of a thrombokinase. Once sufficient thrombokinase is formed, the conversion of prothrombin to thrombin by thrombokinase and the conversion of fibrinogen to fibrin by thrombin proceed comparatively rapidly. The time required for the thrombokinase-forming reactions determines the blood coagulation time. The nature and sequences in the interaction between the various factors, yielding thrombokinase, have yet to be elucidated. It is known that AHF and PAcG are

‡ Following the proposal of Milstone(11), thrombokinase is defined as an agent which has the capacity to convert prothrombin to thrombin directly.

§ Biggs defines thromboplastin as a substance which is directly responsible for conversion of prothrombin to thrombin in the presence of calcium ions.

‖ Plasma thromboplastin component is assumed to be identical with the Christmas factor(12).

utilized during the clotting of blood. Whether they become a component part of the thrombokinase complex is at present not known. According to Owren and his associates(17) convertin (obtained by the interaction of brain extract and proconvertin, present in serum or plasma eluate) and accelerin¶ combine to form prothrombinase (thrombokinase) which in the presence of calcium produces rapid conversion of prothrombin to thrombin. Whether the calcium necessary for the formation of thrombokinase becomes an integral component of the thrombokinase complex or whether it serves merely as a catalyst is not known.

Summary. Plasma accelerator globulin, antihemophilic factor as well as serum eluate were found to produce increased thrombin elaboration in a reaction mixture consisting of bovine plasma eluate, platelets and calcium ions. In the presence of both plasma accelerator globulin and antihemophilic factor, conversion of prothrombin to thrombin became accelerated. Addition of serum eluate to the plasma eluate enhanced the accelerating influence of either plasma accelerator globulin, of antihemophilic factor or of both in the utilization of prothrombin. Upon preincubation of those factors which are supposed to participate in the conversion of prothrombin, before addition of a plasma eluate to the incubation mixture, it was found that more prothrombin was converted in a shorter period of time than in the one-stage thrombin formation procedure. The greater velocity of thrombin formation in the two-stage procedure indicated that precursory interactions of certain factors had taken place during the initial incubation. Evidence has been obtained that antihemophilic factor as well as plasma accelerator globulin will interact with platelets and calcium ions only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity.

¶ Obtained by addition of thrombin to prothrombin and proconvertin free bovine plasma. Accelerin was then precipitated by dilution and acidification. The preparation may have been contaminated with AHF.

1. Alexander, B., *New England J. Med.*, 1955, v252, 432.

2. Aggeler, P. M., White, S. G., Glendening, M. B., Page, E. W., Leake, T. B., and Bates, G., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 692.

3. Campbell, E. W., and Stefanini, M., *ibid.*, 1953, v83, 105.

4. Graham, J. B., Langdell, R. D., Morrison, Jr., F. C., and Brinkhous, K. M., *ibid.*, 1954, v87, 45.

5. Seegers, W. H., Johnson, S. A., Fell, C., and Alkjaersig, N., *Am. J. Physiol.*, 1954, v178, 1.

6. Lewis, M. L., and Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 636.

7. Graham, J. B., Penick, G. D., and Brinkhous, K. M., *Am. J. Physiol.*, 1951, v164, 710.

8. Langdell, R. D., Wagner, R. H., and Brinkhous, K. M., *J. Lab. Clin. Med.*, 1953, v41, 637.

9. Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.*, 1947, v13, 231.

10. Jensen, H., Gray, E. J., and Schaefer, Jr., E. H., *Acta Haemat.*, 1955, v13, 377.

11. Milstone, J. H., *Medicine*, 1952, v31, 411.

12. Biggs, R., *Brit. Med. Bull.*, 1955, v11, 5.

13. Spurling, C. L., and King, P. D. L., *J. Lab. and Clin. Med.*, 1954, v44, 336.

14. Flynn, J. E., and Coon, R. W., *Am. J. Physiol.*, 1953, v175, 289.

15. Seegers, W. H., *Coagulation of Blood. Advances in Enzymology*, 1955, v16, 23. Interscience Publishers Inc., New York.

16. Mann, F. D., and Hurn, M. M., *Am. J. Physiol.*, 1953, v175, 65.

17. Owren, P. A., Rapaport, L. I., and Aas, K., *Sang*, 1954, v25, 752.

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Influence of Cortisone on Experimental Viral Infection. II. Effects on Antibody Formation and Acquired Immunity.* (22136)

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It has been demonstrated that infection of a number of mammalian species with a variety of microorganisms is altered to the detriment of the host by cortisone(see reviews(1,2)). It has also been established with less unanimity that the formation of humoral antibody to non-replicating antigens is suppressed by the administration of cortisone(3,4). However, few studies(5,6) have been published of the influence of cortisone on the development of circulating antibody during actual infection. Perhaps the rapidly lethal outcome of most experimental infections studied precludes the formation of detectable antibody unless antimicrobial therapy is used to arrest infection (5,6).

In the present study, 2 avirulent influenza A virus strains capable of multiplication to high titer without the concomitants of pneumonia or death have been used to permit study of antibody formation resulting from unrestricted multiplication of a viable antigen. In a companion experiment, study has been made of the effectiveness of the immunity so acquired in resisting challenge with a virulent strain of one of the viruses used in immunization.

Materials and methods. Mice. CFW mice which averaged 20 g in weight were employed in groups of 6-21 as indicated in the protocols. Mice were inoculated intranasally with viral suspensions while under light ether anesthesia. Blood for antibody determinations was removed under chloroform anesthesia by cardiac puncture exsanguination. *Viruses.* The 1947 FM-1 influenza A strain was employed in two forms: 1) as unadapted first mouse lung passage virus (10% mouse lung suspension), and 2) as a 10% mouse lung suspension of 29th passage mouse-adapted virus. A 1950 non-mouse-adapted influenza A virus (Rockefeller Institute JB strain)(7) was also employed as undiluted allantoic fluid. *Cortisone.* Cortisone was used in the form of cortisone acetate (Cortone® Merck); the aqueous vehicle used in its suspension was employed for the subcutaneous injection of control animals.‡ *RDE.* The filtrate of a 48-hour neopeptone broth culture which had been inoculated with the Inaba strain of *Vibrio comma* and incubated at 37°C was used as receptor destroying enzyme to remove serum inhibitor. *Viral titrations.* Assay of pulmonary virus was accomplished by inoculation of four 10-day-old White Leghorn embryos with the indicated dilution of mouse lung. Allantoic fluids were harvested after 40-48 hour incubation at 35°C and titered for hemagglutinin at 1:4 dilution with 1% human "O" RBC. MID_{50} (mouse infective dose) was determined as previously described(7). *Scoring of pulmonary lesions.* When it became apparent that cortisone did not kill control mice under the conditions employed, the conventional maximum score method(8) (which considers mortality) was used in grading pulmonary lesions, but with a percentile expression of results to facilitate comparison among mouse groups of varying size. *Antibody titrations.* Sera were heated at 56°C for 30 minutes prior to their use. Neutralizing antibody was measured by the mouse-egg technic which can determine neutralization in mice of non-mouse-adapted virus (7). Hemagglutination-inhibiting antibody was measured by a method described earlier (9) after prior incubation of sera with equal volumes of RDE for 18 hours. Human "O" RBC in initial concentrations of 1% were used.

Results. Effect of cortisone on formation of antibody in mice infected with influenza A

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‡ These preparations were generously supplied by Merck & Co., Rahway, N. J.

TABLE I. Effect of Varying Doses of Cortisone on Formation of Neutralizing Antibody in Mice Infected with Influenza A Virus (JB Strain).

Serum from mice inj. with:		Neutralizing antibody titer v.s.	
Cortisone (mg)	Virus	50 MID_{50}*	5000 MID_{50}
0	+	>32†	12
5	+	>32	12
1	+	>32	6
2.5 q 3 days × 6	+	6	<4
5	0	<4	<4

* Mouse infectious doses.
† Reciprocal of serum dilution 50% neutralization.

viruses. Formation of neutralizing or hemagglutination-inhibiting antibody was suppressed during infection of cortisone-injected mice with either of 2 strains of influenza A virus. In one experiment groups of 18-21 mice were killed with chloroform and bled from the heart 19 days following the intranasal inoculation of the JB strain of influenza A virus and the administration of cortisone or control material. The results of titrations of serum neutralizing antibody are presented in Table I. Definite suppression of neutralizing antibody formation followed prolonged cortisone dosage initiated on the day of infection, but was not evident after single injections of 1 and 5 mg of cortisone administered at the time of viral inoculation.

In a second experiment, mice were inoculated with unadapted FM-1 (1947) virus, then subjected to either of two cortisone regimens, as shown in Table II. One schedule consisted of administration of 5 mg doses on the day preceding, the day of, and the day after virus inoculation; the second schedule involved the protracted administration of smaller doses as used in the first experiment. Pooled sera (15 mice/group) were obtained on the 18th day and titrated for hemagglutination-inhibiting antibody. Results are shown in Table II. As in the first experiment, only prolonged administration of cortisone inhibited development of antibody.

Effect of cortisone on acquired active immunity against FM-1 virus. Six groups of 6 mice each were inoculated intranasally with avirulent non-adapted FM-1 virus or saline (Table III). Fourteen days later mice were challenged with mouse-virulent FM-1. Mice received concomitant injections of cortisone as indicated. No fatalities occurred in mice previously infected with FM-1, indicating that such mice possessed at least partial immunity when compared with mice not previously infected with virus. However, pulmonary lesions involving 33% of total lung volume were found in mice given large amounts (15 mg) of cortisone at the time of challenge. It is also of interest that two groups of mice given cortisone (5 or 15 mg) had demonstrable pulmonary virus 7 days after challenge, in contrast to mice injected with saline, who were found to have none. Thus, cortisone in large doses may lower acquired host resistance to allow viral multiplication, and result in the appearance of manifest disease, as well.

Discussion. To the author's knowledge the present study is the only published investigation which describes inhibition by cortisone of antibody formation during infection. A study by Kass *et al.*(10) with a virulent influenza A strain failed to demonstrate humoral antibody in either control or cortisone-treated mice 6 days after inoculation—leaving the question of antibody inhibition unanswered. Studies of streptococcal infection in rabbits(5) and man(6) have shown no suppression of antistreptolysin O formation with cortisone. Germuth *et al.*(11) found no interference by cortisone with the development of an immune response to pneumococcal infection of the rabbit, but made no study of circulating antibody.

The demonstration in the present study

TABLE II. Effect of Varying Doses of Cortisone on Formation of Hemagglutination-Inhibiting Antibodies in Mice Infected with Influenza A (FM-1) Virus.

Serum from mice inj. with:		
Cortisone (mg)	Virus*	Antibody titers†
0	+	64
5 × 3	+	32
0	0	<8
5 × 3	0	<8
2.5 q 3d × 6	+	<8

* 10^3 EID_{50}.
† Reciprocal of highest dilution of serum which inhibited hemagglutination with 8 hemagglutinating units of virus.

TABLE III. Effect of Cortisone on Aquired Active Immunity against FM-1 Virus; Challenge of Immunized Mice with Virulent Virus.

Day 1	Day 14		Day 21		
Initial inoculation	Challenge inoculum*	Cortisone (mg)	D/T†	Lesions, maximum score (%)	Virus‡ in lungs
FM-1 (1st mouse pass.)	FM-1 (29th pass.)	5	0/6	10	+
"	"	0	"	0	0
"	"	5 × 3§	"	33	+
Saline	FM-1 (29th pass.)	0	4/6	72	
"	"	5	4/5	80	
FM-1 (1st mouse pass.)	Saline	0	0/6	3	0

* 10^5 MID_{50}. † No. of dead mice/total in group. ‡ +, 10^{-2} lung suspension infects eggs; 0, 10^{-2} lung suspension does not infect eggs. § On days 13, 14, 15.

that antibody suppression is dependent on cortisone dosage suggests an explanation for the inhibition of antibody formation noted here but not observed by others. Species difference would not appear to be an important factor in view of evidence of profound inhibition by cortisone in the rabbit of antibody formation to non-multiplying antigens(3,4).

The present study has demonstrated that cortisone may suppress the formation of antibody during the course of a viral infection, and further, that the immunity conferred by infection may be partially surmounted by administration of cortisone with a challenge inoculum. However, reduction in pre-existing acquired immunity is effected by cortisone dosages inadequate to suppress antibody formation. Thus, the persistence of pulmonary virus and the induction of pneumonia in immune mice given cortisone cannot be ascribed to effects on circulating antibody. These findings are in accord with previous observations that 1) lethal infections may be rapidly induced with cortisone in naturally resistant hosts(5,12) and 2) that increased viral multiplication and mortality may be effected by cortisone in a host (the chick embryo) incapable of demonstrable antibody formation(13).

It is conceivable that the demonstrated multiplication or persistence of virus in an immune host may be explicable on the basis of cortisone-induced reactivation of pre-existing pulmonary virus, but thus far the phenomenon of viral reactivation with cortisone has been studied only in the chick embryo(14).

Summary. 1) The formation of neutralizing and hemagglutination-inhibiting antibodies was suppressed by cortisone administered during infection of mice with influenza A viruses. 2) Suppression of antibody was dependent on prolonged administration of cortisone and was not effected by large doses given at the onset of infection. 3) Acquired active immunity induced by infection of mice with avirulent virus was partially surmounted by administration of cortisone with a challenge inoculum of virulent virus. 4) Cortisone in doses insufficient to suppress antibody formation induced lesions and persistence of pulmonary virus following challenge of mice with previously acquired immunity.

1. Thomas, L., *Ann. Rev. Med.*, 1952, v3, 1.
2. Shwartzman, G., *Symposium N. Y. Acad. Med.*, Columbia University Press, N. Y., 1953.
3. Germuth, F. G., Jr., and Ottinger, B., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 815.
4. Bjorneboe, M., Fischel, E. E., and Stoerk, H. C., *J. Exp. Med.*, 1951, v93, 37.
5. Mogabgab, W. J., and Thomas, L., *J. Lab. and Clin. Med.*, 1952, v39, 271.
6. Hahn, E. O., Houser, H. B., Rammelkamp, C. H., Jr., Denny, F. W., and Wanamaker, L. W, *J. Clin. Invest.*, 1951, v30, 274.
7. Kilbourne, E. D., and Horsfall, F. L., Jr., *J. Immunol.*, 1951, v67, 431.
8. Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, v70, 209.
9. Walker, D. L., and Horsfall, F. L., Jr., *ibid*, 1950, v91, 65.
10. Kass, E. H., Lundgren, M. M., and Finland, M., *ibid.*, 1954, v99, 89.
11. Germuth, F. G., Jr., Ottinger, B., and Oyama, J., *Bull. Johns Hopkins Hosp.*, 1952, v91, 22.
12. Kilbourne, E. D., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 135.
13. Kilbourne, E. D., *J. Immunol.*, 1955, v74, 57.
14. ———, *J. Exp. Med.*, 1955, v101, 437.

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Plasma Penicillin Levels from Oral Penicillins V and G and Intramuscular Penicillin G.* (22137)

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(With technical assistance of Clare Wilcox and Ann Najarian.)

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Penicillin V (phenoxymethyl penicillin), because of its stability at low pH ranges, its low solubility in an acid medium and rapid conversion to the water soluble salt at neutral or alkaline pH, has been suggested as possibly superior to penicillin G for oral administration(1-3). The available information concerning penicillin V was reviewed recently by Jones and Finland(4) who, in a controlled study, compared the penicillin activity in the plasma of normal subjects following ingestion of single tablets of 200,000 units of penicillins G and V. They found that penicillin V produced higher and more prolonged blood levels than did penicillin G as judged by the antistreptococcal and antistaphylococcal activity of the plasma, particularly when the doses were taken after a meal. They also showed that the numerical value assigned to the concentration of penicillin in plasma depends on the strain used in the assay and on the type of penicillin (*i.e.*, V or G) in the standard solution employed as the control from which the concentrations are computed. Wright *et al.*(5) also showed that differences found by microbiological assay methods depend mainly on the assay organism but also on the medium used for the dilutions. They too found considerably higher blood concentrations from penicillin V than from the same 200,000 unit dose of penicillin G given orally, and, in addition, they showed that twice as much of penicillin V is excreted as the active form in the urine.

The present paper presents further comparisons of levels of penicillin in plasma resulting from the same amounts of penicillins V and G given orally at various dosage levels, and these, in turn, are compared with levels obtained from intramuscular injection of the same amount of penicillin G.

Materials and methods. The subjects were 8 normal young men and 24 ward patients, 3 of whom were women; their ages ranged from 15 to 86 (avg 50 yr) and their weights ranged from 40 to 95 kg (avg 66 kg). None had recently received any antimicrobial agents. All had grossly normal renal function as judged by routine clinical laboratory tests except one who had renal insufficiency with nonprotein nitrogen levels ranging as high as 145 mg%. All were able to and did ingest normal quantities of food and liquids, but the intakes were not controlled. Three dosage forms of penicillin were used, *viz.* (1) the free acid of penicillin V in 200000 unit tablets, (2) buffered potassium penicillin G ("Pentids," Squibb) in 200000 unit tablets, and (3) aqueous potassium penicillin G intramuscularly. These were given at 3 dosage levels, *viz.* 200000 units, 400000 units and 1000000 units; individual doses were separated by at least 48 hours. Every dose was given ½ hr after breakfast. All subjects received both penicillin V and G orally at least at one and the same dosage level and many also received an i.m. injection of the same number of units of penicillin G. Three of the young men received all 3 dosage forms at all 3 levels, and 5 subjects, including these 3, received all 3 dosage forms at each of the 3 levels. Assays for penicillin in plasma were done by the 2-fold dilution method in broth as in the previous study(4) except that only *Strep. 98* was used as the assay organism and the numerical values assigned were based only on comparisons with a standard of penicillin G. As in the previous study(4), graphic comparisons will be based only on the maxi-

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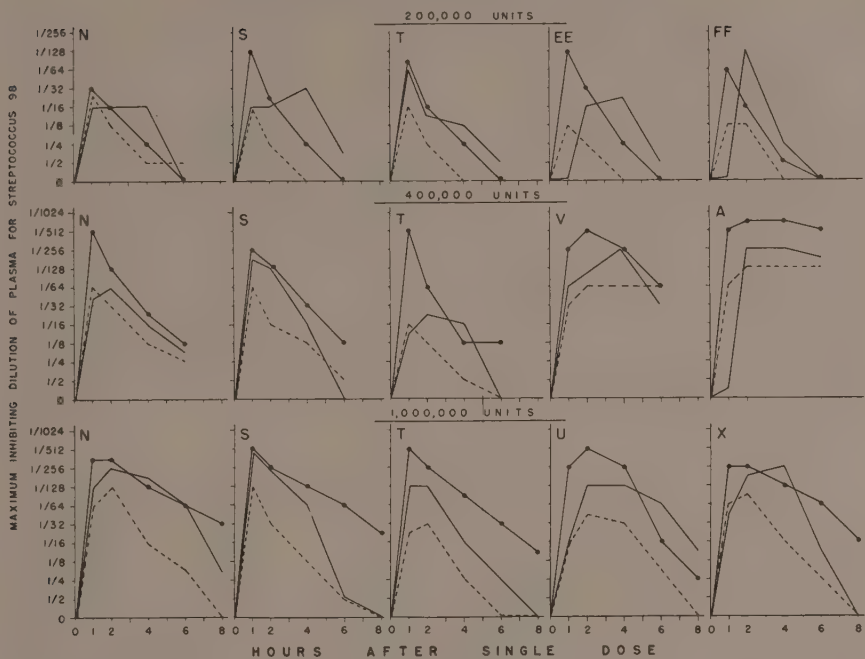

FIG. 1. Plasma penicillin levels in same subjects after same dose of penicillin given as: Penicillin V orally (——), buffered penicillin G orally (– – –), and penicillin G intramuscularly (•——•).

mum dilutions of plasma which inhibit *Strep. 98;* in these studies the standard solution of 1 μg/ml of penicillin G inhibited the test strain at a dilution of 1:128, and 1 μg of the solution of penicillin V inhibited at 1:64. Blood for the assays was obtained before each dose and 1, 2, 4 and 6 hours after the dose in every instance, and also 8 hours after the 1 million unit dose. All specimens of plasma taken immediately prior to a dose showed no detectable penicillin activity.

Results. Comparative Levels in the Same Subject. The levels of antistreptococcal activity obtained in the plasmas of 5 subjects who received all 3 dosage forms at the same dosage level are shown graphically in Fig. 1. The letters in each panel identify the subjects. It is seen from this figure the relative levels of penicillin activity in plasma obtained from the 3 dosage forms varied with the dose. In general, i. m. penicillin G produced higher peak levels more rapidly than the other forms. With the dose of 200000 units these were not as well sustained as from oral penicillin V, whereas with the higher doses they were bet-

TABLE I. Average Concentrations of Penicillin in the Same 5 Subjects after the Same Dose of Penicillin Given in 3 Forms.

Dose units* (× 1000)	Hr after dose	Dosage form: Penicillin G, orally	Penicillin V, orally	Penicillin G, intramuse.
		Avg level† in plasma, μg/ml		
200	1	.22	.21	1.63
	2	.12	.35	.39
	4	.018	.19	.06
	6	.005	.046	0
400	1	.99	.47	7.5
	2	.93	1.8	3.97
	4	.40	1.05	1.40
	6	.20	.22	.70
1000	1	1.01	1.16	7.0
	2	1.4	2.69	6.07
	4	.39	1.40	2.53
	6	.11	.29	.82
	8	.05	.08	.26

* 200000 units = approximately 120 mg penicillin, either as G or V.

† Determined by broth-dilution method using Streptococcus 98, and comparing with a penicillin G standard solution.

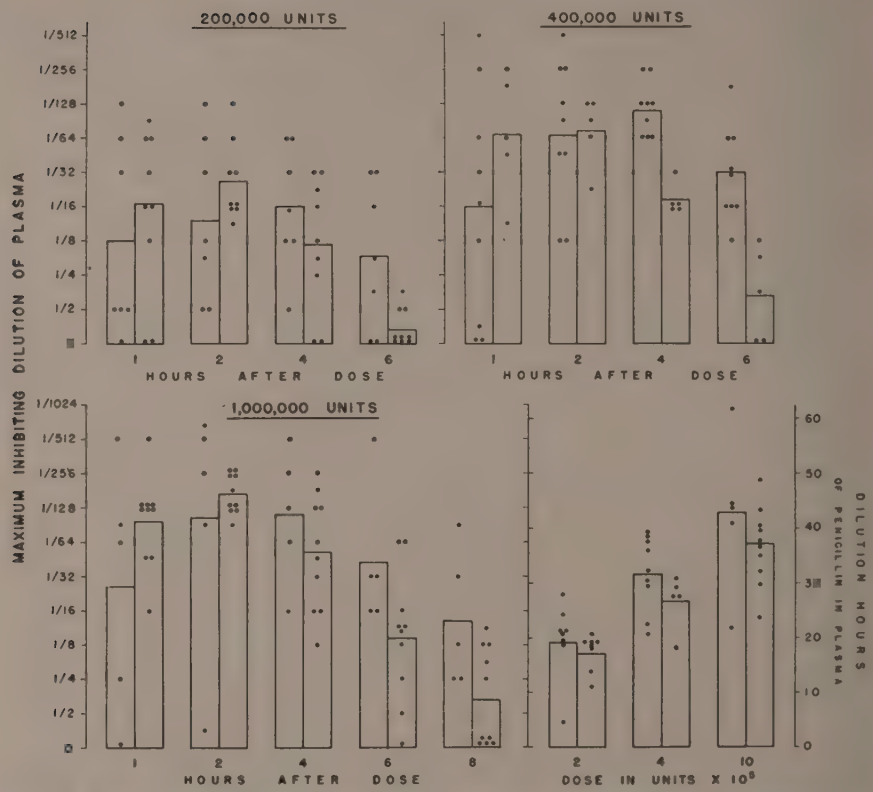

FIG. 2. Effect of age on levels of penicillin in plasma after oral penicillin V. Subjects 60 yr old and over (left) compared with those under 60 (right).

ter sustained and at higher levels. Calculations of the areas ("dilution-hours") under the curves following the 200000 unit doses showed no significant difference between oral penicillin V and i.m. penicillin G ($P = >0.1$), but the differences between both of these and the values for oral penicillin G were significant ($P = <0.02$ in each instance). After the 400000 unit doses, the levels were more or less uniformly higher with i. m. penicillin G than after oral penicillin V, and these in turn were higher than those following oral penicillin G. Calculations of the areas under these curves, however, yield values that are not statistically significant; this is due, in large part, to the fact that plasma-penicillin levels were not obtained after 6 hours and these are not readily extrapolated. On the other hand, 1 million units of i. m. penicillin G gave the highest levels of penicillin activity throughout, and oral penicillin V yielded higher levels than oral penicillin G. In this instance, the differences between the areas under the curves are statistically significant in every instance.

The average plasma penicillin levels, calculated as penicillin G, are shown in Table I; they show the same differences that are reflected in Fig. 1.

Effect of Age. Since a number of the subjects were in the older age group, an attempt was made to determine the effect of this factor on the levels of penicillin in the blood. This was done only for oral penicillin V. The data are charted in Fig. 2. For all 3 dosage levels, the penicillin activity in the plasma of the subjects over 60 years old rose more slowly, achieved a peak later, and thereafter

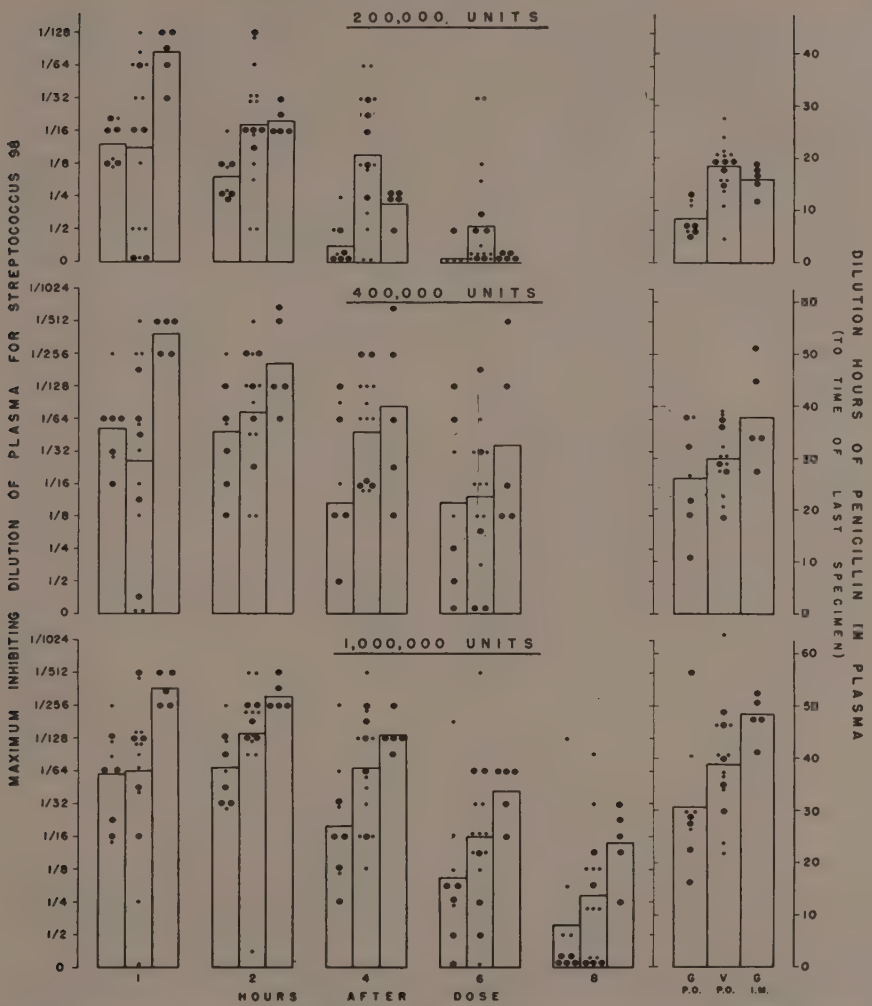

FIG. 3. Penicillin levels in plasma following administration of penicillin V orally (center) compared with penicillin G, orally (left) and intramuscularly (right). The large dots represent subjects who received all 3 dosage forms at the stated intervals.

were sustained better than in the younger ones. This may be a reflection of differences in renal function. This figure also shows the areas (dilution-hours) under the curves of the plasma penicillin levels; these values and their means were higher in the older subjects at each of the 3 dosage levels.

Further Comparisons of the 3 Dosage Forms. The levels of penicillin in plasma obtained in all the subjects studied and the mean values for each dosage form at each dose level are charted in Fig. 3. In this figure the large dots represent the values in the subjects for whom data are available for all 3 forms at the stated dose levels. At the right are also shown the areas or "dilution-hours" under the curves of the plasma penicillin levels and the means of these values. Here again it is seen that penicillin V is superior to penicillin G at each dose level. The dose of

200000 units of i. m. penicillin G gave higher and earlier peak levels but these were less sustained than from oral penicillin V; the average number of dilution-hours, however, was quite similar for these 2 forms at this level, and these were both greater than from oral penicillin G. After doses of 400000 units and 1000000 units, on the other hand, i. m. penicillin G gave the highest and best sustained levels and oral penicillin V gave higher and better sustained levels than oral penicillin G; these differences are reflected in the number of dilution hours of penicillin in the plasma.

Summary. Oral penicillin V gave higher and better sustained levels of penicillin activity in the plasma than oral buffered potassium penicillin G at each of 3 dosage levels, *viz.* 200000, 400000 and 1 million units. Intramuscular penicillin G yielded higher and better sustained levels than oral penicillin V in equivalent doses given at levels of 400000 or 1 million units. An i. m. dose of 200000 units of penicillin G produced higher peak levels and these occurred earlier but they were less well sustained than with this amount of oral penicillin V; the total amount of penicillin absorbed from this amount of penicillin was not significantly different for these 2 dosage forms. Persons over 60 years of age attained peak levels later; in general they had higher and better sustained levels of penicillin in the plasma from any given dose than did younger individuals.

1. Brandl, E., Giovannini, M., and Margreiter, H., *Wien. med. Wchnschr.*, 1953, v103, 602.

2. Giovannini, M., *ibid.*, 1954, v104, 910.

3. Brandl, E., and Margreiter, H., *Oesterr. Chem.-Zeitung*, 1954, v55, 12.

4. Jones, W. F., Jr., and Finland, M., *New England J. Med.*, 1955, v253, 754.

5. Wright, W. W., Kirshbaum, A., Arret, B., Putnam, L. E., and Welch, H., *Antibiotic Med.*, 1955, v1, 490.

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Effect of Dietary Protein and Energy on Chick Liver Fat Accumulation.*† (22138)

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Best and Huntsman(1) discovered that a dietary deficiency of choline resulted in an excessive accumulation of fat in rat livers. Later work, Harper *et al.*(2) indicated that such compounds as glycine, serine, methionine and betaine, which may function as choline precursors, are able to reduce the level of liver fat in rats on choline-free diets. Channon and Wilkinson(3) and Best and Huntsman(4) suggested that the deposition of liver fat might

TABLE I. Basal Diet for Preliminary Period.

Ingredient	%
Glucose	54.4
Soy protein*	31.6
Soybean oil	4.0
Cellulose	3.0
Methionine	.5
Mineral mix†	5.5
Vit.-antibiotic mix‡	1.0
Total	100.0

* Drackett Assay Protein C-1.

† Provides per lb diet: NaCl 0.5%, Ca 1.6%, P 0.88%, K 1.3 g, Zn 1.08 mg, Mn 33.3 mg, Fe 30.6 mg, Co 0.6 mg, Cu 1.2 mg, Mg 300 mg, and I 5.0 mg.

‡ Provides per lb diet: Vit. A 4000 I.U., Vit. D_3 500 I.C.U., alpha-tocopherol 7.0 mg, menadione 0.6 mg, choline Cl 868 mg, inositol 455 mg, niacin 45 mg, calcium pantothenate 10 mg, riboflavin 3.6 mg, pyridoxine-HCl 2.7 mg, thianine-HCl 1.8 mg, folic acid 1.4 mg, p-amino benzoic acid 0.9 mg, biotin 90 μg, vit. B_{12} 6 μg, penicillin 4 mg.

* Part of thesis by senior author in partial fulfillment of requirements for degree Doctor of Philosophy, Iowa State College.

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TABLE II. Experimental Diets.

Ingredient	Calculated dietary protein level 14	18	22	26	30	14	18	22	26	30
	Low energy*					High energy†				
Glucose	52.3	47.6	42.9	38.3	33.5	66.6	61.6	56.4	51.4	46.2
Soy protein	16.4	21.2	26.1	30.9	35.8	16.4	21.2	26.1	30.9	35.8
Soybean oil	1.0	1.0	1.0	1.0	1.0	10.0	10.2	10.5	10.7	11.0
Cellulose	23.3	23.2	23.0	22.8	22.7	—	—	—	—	—
Methionine	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Mineral mix‡	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Vit.-antibiotic mix§	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Calculated to contain 720 C/lb productive energy.
† " " " 1055 C/lb " " .
‡ Minerals supplied as in Table I.
§ Vitamins and antibiotic supplied as in Table I.

be linked to protein metabolism. In this respect Eckstein(5) found that only methionine exhibited a lipotropic effect when each of the 10 essential amino acids was incorporated singly into a choline-free diet. Threonine(6) has been shown to exhibit a lipotropic effect when incorporated into a casein diet complete in all known nutrients, but this phenomenon may have occurred because of the low biological availability of threonine in casein(7). The data of Winje *et al.*(8) support this conception. Recently Lucas and Ridout(9) substantiated the suggestion of Harper *et al.*(2) that the level of protein in the diet has an influence on the accumulation of fat in the livers of rats. They reported a larger percentage of fat in the livers of rats on methionine-supplemented low-protein diets than in the livers of rats on diets higher in protein. As far as the authors are aware, no such effect on liver fat has previously been reported for the chick.

The purpose of this investigation was to study the effect of dietary protein and energy levels on the accumulation of fat in the livers of chicks.

Methods and materials. Eighty day-old chicks were maintained on a nutritionally complete basal diet (Table I) for 10 days after which they were randomly assigned to 40 pens containing 2 birds per pen. At this time 4 pens were allotted to each of the 10 experimental diets (Table II) under a restricted randomized block design. The experimental feed, except that which was in the feeders, was kept refrigerated at 38°F during the experimental period. The birds were maintained in thermostatically controlled batteries with feed and water supplied *ad libitum* during the experimental period. After 10 days on the experimental diet the birds were sacrificed and their livers were removed and frozen at –20°F. The livers from the birds in each pen were pooled, washed of adhering blood and fatty tissue and blended in a Waring Blendor. The resulting homogenous mixture was dried for 16 hours at 212°F and then ground for analysis. Crude fat determinations were made according to the A.O.A.C.(10) method.

Results. The data in Table III indicate that the energy level of the diet apparently had no marked effect on the chick liver fat. A statistical analysis of the data indicates a significant linear response and a significant quadratic response in percent liver fat due to dietary protein level changes on the high energy diets and the low energy diets, respectively. As shown in Fig. 1, this suggests that the amount of fat in the liver is inversely related to the protein level of the diet.

Discussion. Chicks consuming a high fat (10%) diet accumulated no more fat in their

TABLE III. Effect of Energy Level of Diet on Response to Protein Level Increments.

Level of protein in diet (%)	Liver fat (%) High energy diet (1055 cal/lb)	Low energy diet (720 cal/lb)
14	13.35	12.35
18	13.76	13.58
22	11.25	13.24
26	10.57	11.79
30	10.20	10.54

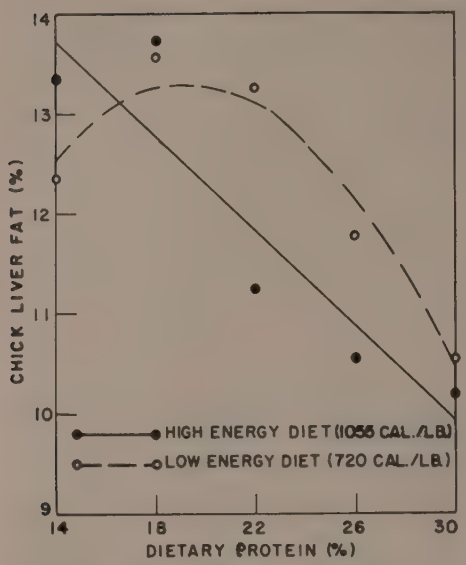

FIG. 1. Effect of high and low energy diets on liver fat content with relation to protein level increments.

livers than those consuming a low fat (1%) diet. This indicates that dietary fat *per se* was not deposited in the chick's liver to any great extent. Harper *et al.*(2) reported a similar effect in rats.

The present work reveals that over a specific (14 to 30%) range there is an inverse relationship between the level of protein in the diet and the amount of fat deposited in the chick's liver. These results are in agreement with those of Lucas and Ridout(9) with rats. The diets in the present study were sufficient in all nutrients known to be required by the chicks, and these, as well as the energy content of the diet, were held approximately constant over the range of dietary protein levels tested. In most of the previous work using the rat, these extraneous variables had not been as closely controlled.

It is apparent from these data that protein exerts a lipotropic effect not directly involving its choline or choline precursor content.

Summary. The present work reveals that over a range of protein levels from 14 to 30% there is an inverse relationship between the level of protein in the diet and the amount of fat deposited in the chick's liver. The dietary energy level does not appear to influence the chick liver fat level.

1. Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1932, v75, 405.

2. Harper, W. J., Monson, W. J., Benton, D. A., Winje, M. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1954, v206, 151.

3. Channon, H. J., and Wilkinson, H., *Biochem. J.*, 1935, v29, 350.

4. Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1935, v83, 255.

5. Eckstein, H. C., *J. Biol. Chem.*, 1952, v195, 167.

6. Singal, S. A., Hazen, S. J., Sydenstricker, V. P., and Littlejohn, J. M., *ibid.*, 1953, v200, 867.

7. Donovan, G. A., and Balloun, S. L., unpublished data.

8. Winje, M. E., Harper, A. E., Benton, D. A., Boldt, R. E., and Elvehjem, C. A., *J. Nutrition*, 1954, v54, 155.

9. Lucas, C. C., and Ridout, J. H., *Can. J. Biochem. Physiol.*, 1955, v33, 25.

10. Association of Official Agricultural Chemists, *Official Methods of Analysis*, 1950, 7th ed.

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Serum Sodium Concentration of Hypertensive Rats: Relation to NaCl Intake, Blood Pressure and Age.* (22139)

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Alterations of sodium metabolism have been studied intensively in both experimental and clinical hypertension. Among the deviations from normal are changes in total body sodium (1), extracellular fluid volume(2), sodium and water contents of aortic walls(3), as well as plasma and serum sodium concentration. With respect to the last, results have ranged from no apparent change in a very small number of experimentally hypertensive rats(1) to slight but definite increases in larger numbers of hypertensive rats(4) and of hypertensive patients(5). In addition, a relative aversion to NaCl, together with polydipsia and polyuria, has been observed in rats made hypertensive by bilateral renal encapsulation with latex envelopes(6-8). The mechanisms by which this relative NaCl aversion is associated with the induced perinephritis and experimental hypertension are still unclear. An elevated serum Na concentration in hypertensive rats might conceivably be one factor in this relative aversion.

With this possibility in view, the studies now reported were done to determine (a) whether serum Na concentration is increased in rats with established hypertension, (b) whether differences in serum Na concentration can be related to known differences in voluntary NaCl intake and/or to systolic blood pressure and (c) whether age at encapsulation affects serum Na concentration. It has been shown previously that age at encapsulation is one of the factors affecting NaCl aversion(8).

Methods. Male rats of the Sprague-Dawley strain, and of similar ages, were kept in individual cages in a thermoregulated room maintained at 25 ± 1°C, illuminated from 8 a.m. to 6 p.m. They were made hypertensive by enclosing both kidneys in latex rubber capsules(9). A group of 31 normal rats (series I) served as controls. In 23 rats (series II) renal encapsulations were done when they were 50 to 90 days old; in 14 rats (series III) when they were 126 to 165 days old. At least 3 months were allowed for development of hypertension(8). All rats were sacrificed when approximately 300 days old. Prior to sacrifice, *ad libitum* intakes of tap water, .15 M NaCl solution and Purina laboratory chow were measured in 27 rats for 5 days as described earlier(8). Blood pressure was measured by microphonic manometer(10) but without anesthesia(7). At the end of the 5th day the rats were lightly anesthetized by ether inhalation and their necks were carefully shaved. On the 7th day groups of normal and hypertensive rats were sacrificed by guillotine decapitation without anesthesia and 5 to 6 ml of blood were collected directly in test tubes by free flow from the neck vessels without anticoagulant. During collection, the shaved skin of the neck was retracted to prevent contamination of the collected blood by Na from skin or hair. The blood was centrifuged, the serum separated and analyzed for Na by a lithium internal standard Baird flame photometer model number DB2. Sera were diluted 1:100 (using pipettes whose delivery volume was individually checked) in order to provide concentrations within the range of maximum accuracy of the photometer (1.0 to 1.5 mEq./L). Dilutions were made with Na-free distilled water in 100 ml volumetric flasks at 22°C. Each serum sample was bracketed by at least 2 standard solutions, with one Na concentration above and one below the unknown. To determine the precision of the photometer and analytic procedure in general, several aqueous test solutions of NaCl were analyzed. Two were prepared gravimetrically from desiccated NaCl by one of us and analyzed as unknowns by another. Two com-

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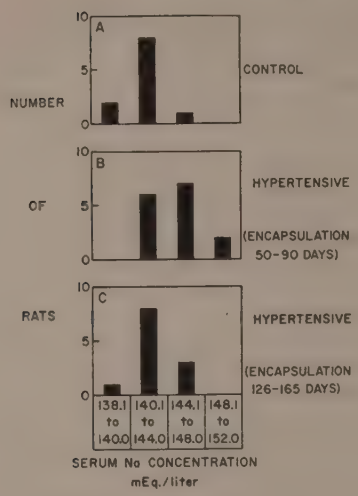

FIG. 1. Frequency distribution of serum Na concentrations (mEq./L) of control and hypertensive rats.

mercially prepared standard solutions‡ of highest accuracy were also analyzed. Typical of these tests are 3 analyses of standards containing 150.3, 100.0 and 5.0 mEq. Na/L which were diluted for analysis 1:100, 1:100, and 1:5 respectively to be in the range of the diluted serum Na. These analyses yielded, in duplicate determinations, final figures of 149.6 and 149.9, 100.0 and 100.0, 5.0 and 5.0 mEq./L respectively. Hence, if each unknown was bracketed by 2 standards, read at least twice and calculated from a separate calibration curve, the precision of the photometer in the range of serum Na concentration was ±0.5 mEq./L. This precision agrees with that reported by others(5). Fisher's test for significance of differences between means was used for statistical comparison of data from any 2 groups(11).

Results. Fig. 1 shows the range and distribution of serum Na concentrations observed in (A) control rats (B) hypertensive rats following encapsulation at 50 to 90 days of age and (C) hypertensive rats following encapsulation at 126 to 165 days of age. Serum Na concentrations tended to be slightly higher than normal in those rats that were made hypertensive by encapsulation while still relatively young (Fig. 1B). As shown in Table I this difference, in terms of mean serum Na concentration, was 2.1 mEq./L. and was statistically significant ($P < .01$). On the other hand, rats made hypertensive by renal encapsulation at 126 to 165 days of age had a mean serum Na concentration which was within the normal range (Fig. 1C and Table I).

The mean *ad libitum* intake of NaCl in the form of .15 M NaCl solution by normal rats in this study was 8.2 ± 1.9 ml/100 g B.W./day. For hypertensive rats encapsulated at 50 to 90 days of age the corresponding figure was 3.0 ± 0.8 ml/100 g B.W./day, showing a highly significant reduction ($P < .01$) in agreement with a number of earlier comparisons(6-8). It is tempting to consider the lower NaCl intake simply a direct effect of the higher serum Na concentration but such an inference is not justifiable. Figure 2 shows the individual intakes of .15 M NaCl solution plotted against serum Na concentrations of the corresponding rats (closed circles for controls, crosses for hypertensive rats). The linear correlation in this plot based on individual rats is not good ($r = -.45$, $P < .05$). Hyperbolic, parabolic, logarithmic and exponential functions did not fit the data any better than the simple linear regression equation. Thus, as a group, rats encapsulated at 50 to 90 days of age presented a significant NaCl aversion together with a slight but significant increase in serum Na concentration. Yet in these same rats, considered singly, no close relationship existed between individual serum Na concentrations and their individual NaCl intakes (Fig. 2).

Mean systolic arterial blood pressures in the 3 groups were 138 (control, Series I), 188 (Series II) and 206 (Series III) mm. Hg respectively. The last two values were significantly higher than the control value ($P < .01$), but did not differ significantly

‡ We are grateful to Mr. Sage and Mr. Lightfoot of Baird Associates, Cambridge, for a sample of their reference standard solution. The second solution was a standard for flame photometry and spectrophotometry prepared by Hartmann Leddon Co., Philadelphia, Pa.

TABLE I. Effect of Age at Encapsulation on Serum Na Concentration and Blood Pressure of Normal and Hypertensive Rats.

	No. of series	Mean age at encapsulation (days)	Mean post-operative age (days)	Mean age at sacrifice (days)	No. of rats	Serum Na conc. (mEq./L)*	P	Systolic blood pres. (mm Hg)	P
Control	I	—	—	350 (268-442)‡	31	143.0 ± .4†		138 ± 2	
Hypertensive	II	63 (50- 90)	246 (160-330)	309 (280-380)	23	145.1 ± .6	<.01	188 ± 7	<.01
	III	150 (126-165)	140 (104-176)	290 (268-345)	14	142.6 ± .7	<.01	206 ± 11	.20

* Each datum can be measured with an accuracy of ± .5 mEq./liter but can be read from a calibration curve with a precision of ± .1 mEq./liter. For purposes of statistical computation the data are tabulated here with 1 more figure than is significant, and the final figure for the mean should be rounded off.

† ± stand. error of the mean.

‡ Range.

from each other (P = .20) and by this criterion the rats of series II and III were equally hypertensive. Yet serum Na concentrations were elevated in one group and normal in the other (Fig. 1, Table I). In both groups plots of individual serum Na concentrations and individual blood pressures (not shown) revealed no close correlation.

Since all groups of rats were sacrificed at approximately the same age, it is evident that those whose kidneys were encapsulated when young were hypertensive somewhat longer than those whose kidneys were encapsulated later in life. Inasmuch as the young group was the one with elevated mean serum Na concentration, a possible relation between duration of hypertension and serum Na concentration was sought (Fig. 3). Earlier observations have shown that blood pressure is maximally elevated in both old and young rats within 70 days after bilateral latex encapsulation of the kidneys and that the earliest rise is detectable within 14 days(8). Therefore, it was possible to express approximately the duration of hypertension by the number of days between the time of operation and the time of sacrifice for both hypertensive groups (Fig. 3). No close correlation could, however, be established between serum Na concentration and the duration of hypertension.

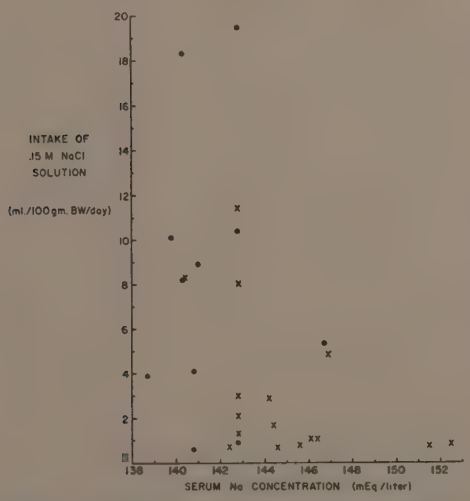

FIG. 2. Individual intakes of .15 M NaCl solution plotted against individual serum Na concentrations of control rats (dot) and rats made hypertensive while young (×).

Discussion. The slight, but significant, elevation of serum Na concentration in rats made hypertensive when young parallels the findings of Holley *et al.*(5) in human essential hypertension and also those of Laramore and Grollman(4) in experimental hypertension in rats with figure of eight ligatures around the kidneys. In the 2 earlier studies, however, age at onset of hypertension and duration of hypertension were not considered with reference to serum Na concentration.

In the present observations, rats whose kidneys were encapsulated when young (50 to 90 days) had a slightly higher mean serum Na concentration while those whose kidneys were encapsulated when older (126 to 165 days) presented a normal distribution of

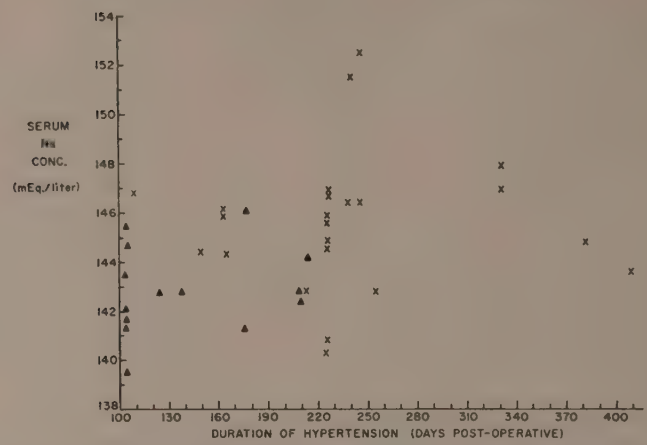

FIG. 3. Serum Na concentration plotted against duration of hypertension for individual rats aged 50 to 90 days (×) and 126 to 165 days (▲) at encapsulation.

serum Na concentrations. A previous study (8) showed that renal encapsulation of younger rats also led to earlier and more definite sodium aversion than did encapsulation later in life. Yet the grade of hypertension was similar with early and late encapsulation. In terms of individual rats, the study reported here has revealed that serum Na concentrations could not be related consistently to the grade of Na aversion, to height of blood pressure, or to duration of hypertension.

It appears, therefore, once more that detailed studies support previous inferences that certain general changes in Na metabolism occur in association with the experimentally hypertensive state under some conditions. Yet a close relationship is not established and further studies are needed to explore possible mechanisms by which the two may conceivably be linked.

Summary. Encapsulating both kidneys of young rats (50 to 90 days) in molded latex envelopes produced hypertension and elevated their mean serum Na concentration slightly (2 mEq./L.) but significantly above that of control rats. The same procedure in older rats (126 to 165 days) produced similar hypertension but mean serum Na concentration remained within normal limits.

The authors are indebted to Miss Cynthia S. Cook for technical assistance and to Mr. Franklin W. Smith for drawing and photographing the figures.

1. Green, R. W., and Sapirstein, L. A., *Am. J. Physiol.*, 1952, v169, 343.

2. Grollman, A., and Shapiro, A. P., *J. Clin. Invest.*, 1953, v32, 312.

3. Tobian, L., Jr., and Binion, J. T., *ibid.*, 1953, v32, 608.

4. Laramore, D. C., and Grollman, A., *Am. J. Physiol.*, 1950, v161, 278.

5. Holley, H. L., Elliott, H. C., Jr., and Holland, C. M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 561.

6. Abrams, M., DeFriez, A. I. C., Tosteson, D. C., and Landis, E. M., *Am. J. Physiol.*, 1949, v156, 233.

7. Fregly, M. J., *Am. J. Physiol.*, 1954, v176, 275.

8. ———, *ibid.*, 1955, v182, 139.

9. Abrams, M., and Sobin, S., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 412.

10. Friedman, M., and Freed, S. C., *ibid.*, 1949, v70, 670.

11. Fisher, R. A., *Statistical Methods for Research Workers*, 10th ed., N. Y., Hafner Pub. Co., Inc., 1948, p. 114.

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Effect of Cholinesterase Inhibitors on Electrolyte Distribution *in vivo*. (22140)

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Mechanisms governing the metabolism of electrolytes at the cellular level remain obscure. It is not known how cells maintain high concentrations of potassium in the face of constant interchange of potassium between intracellular and extracellular compartments. Potassium metabolism may be related to both carbohydrate and acetylcholine metabolism. Observations concerning potassium and acetylcholine have been made *in vitro* on red cells, nerve and muscle. Greig and Holland reported increased permeability of dog erythrocytes due to cholinesterase inhibitors(1). They suggested that cholinesterase activity maintained the selective permeability(2). Other investigators found no direct correlation between inhibition of cholinesterase activity and breakdown of the cellular potassium concentration gradient in the human red cell(3). Recently it has been shown that this erythrocyte synthesizes insufficient acetylcholine to provide energy for accumulation of potassium(4). The relationship between cholinesterase and potassium during neural conduction or muscular contraction would appear to be even more complex. Nachmansohn concluded that acetylcholine increased permeability of nerve membranes (5). Cholinesterase inhibitors increase the rate of entry of sodium into squid axon and decrease that of potassium(6,7). Holland, Dunn and Greig found that acetylcholine caused a loss of potassium from auricular muscle which is reversed by physostigmine (8). They felt that the metabolism of acetylcholine may be correlated with the transfer of sodium and potassium. However, Fenn and co-workers found that acetylcholine caused a small liberation of potassium from muscle, probably due to contraction, and concluded that acetylcholine did not control potassium transfer(9). Little information is available concerning *in vivo* interrelationships of cholinesterase and potassium. In acute experiments cholinesterase inhibitors in lethal doses cause hyperkalemia(10). However, this occurs in association with anoxemia and acidosis. It was felt that repeated sublethal doses of cholinesterase inhibitors would obviate these alterations and, at the same time, induce chronic disturbance of acetylcholine metabolism. It was not known if the loss of cellular potassium due to cholinesterase inhibitors *in vitro* could be reproduced *in vivo*. If this occurred a diet deficient in potassium would impose an impediment to the replenishment of cellular potassium. The following studies were carried out to determine the effect of cholinesterase inhibitors on tissue electrolytes *in vivo*.

Methods. Male Wistar rats weighing 100 g were given a low potassium diet (0.5 mM potassium per 100 g diet) composed of casein, 24%; glucose, 62%; corn oil, 8%; and salt mixture, 6%(11), plus a daily vitamin supplement of 1/100th of a capsule of Tycopan (Lilly). Distilled water was partaken *ad lib*. Additional rats were given sufficient supplementary potassium (as KCl) to bring the potassium content of their diet to 0.4%; these are referred to as normal potassium rats. Anticholinesterase compounds were administered to potassium deficient and normal potassium rats as follows: Group A1 was composed of control rats injected with 0.5 ml isotonic saline and rats injected with 0.05 mg diisopropylfluorophosphate (DFP) in 0.5 ml isotonic saline; and Group B was composed of potassium deficient controls injected with 0.5 ml propylene glycol and rats on both types of diet injected with 0.15 mg p-nitrophenyldiethylthionophosphate (Parathion) in 0.5 ml propylene glycol. In these groups subcutaneous injections were given twice weekly starting 27th day of diet. Rats were sacrificed on 44th, 49th and 71st days. An addi-

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tional series of potassium-deficient rats (Group A2) was composed of controls given 0.5 ml isotonic saline and rats given 0.05 mg DFP twice weekly for 8 weeks, then 0.10 mg twice weekly for 2 weeks, then a final dose of 0.25 mg. The last injection was followed immediately by muscular tremors. This group of rats was injected starting the 5th day of diet and sacrificed on 76th day. No rat was sacrificed prior to 24 hours after an injection. Rats were weighed weekly. Red cell cholinesterase determinations were done by the method of Michel(12) on blood obtained from the tip of tail. Rats were killed by a blow on head, blood obtained by cardiac puncture, and samples of thigh muscle and liver were removed. The hematocrit; plasma water, sodium, potassium and chloride; and skeletal muscle and liver water, fat, potassium, sodium and chloride were determined according to previously described methods (13), except that sodium and potassium were determined by flame photometry.

Results. Body weight. After the first 10 days of potassium-deficient diet the rats had a decreased rate of gain in weight compared with those on normal potassium diet. Administration of DFP and Parathion did not alter rate of gain.

Cholinesterase activity. Fig. 1 shows red cell cholinesterase activity. Rats given Parathion had a moderate reduction in cholinesterase activity and those given DFP had a marked reduction. There was no apparent difference between rats on a potassium deficient diet and those on normal potassium intake. Relative cholinesterase activity of intercostal muscle was estimated histochemically in Group A2(14). Cholinesterase activity at the motor endplate of potassium deficient rats showed no detectable difference from that of normals, but was definitely less in rats given DFP.

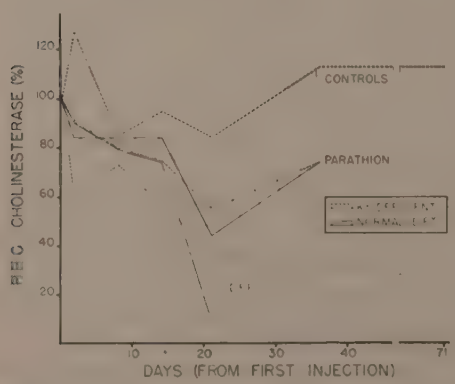

FIG. 1. Cholinesterase levels of red cells in rats given repeated injections of cholinesterase inhibitors (values expressed as % of the mean of 11 determinations on control potassium-deficient rats).

Hematocrits. There were no consistent differences among hematocrit values.

Plasma electrolytes. The potassium deficient diet produced marked hypokalemia and hypochloremia (Table I). There were no significant differences in the plasma electrolytes as a result of the administration of cholinesterase inhibitors.

Muscle water and electrolytes. Skeletal muscle of all groups showed essentially the same water content (Table I). Potassium deficient rats had a marked reduction of potassium in their muscles and a less marked increase in sodium concentration. No differences are found in muscle water or electrolytes between control rats and those given DFP or Parathion.

Liver water and electrolytes. Livers of potassium deficient rats tended to have a higher water content than did the normals (Table I). The reduction in liver potassium in potassium deficient rats was much less than the reduction in muscle potassium and the increase in sodium was slight. There were no apparent differences in liver water and electrolytes between control rats and those intoxicated with anticholinesterase compounds.

Derived data. Extracellular water phase, intracellular water phase, and intracellular sodium concentration are also shown in Table I. These have been calculated as presented by Cotlove *et al.* Changes in skeletal muscle due to potassium depletion, namely, increase in extracellular water, decrease in intracellular phase, and a marked gain in intracellular sodium concentration are similar to those reported by these authors(15). These changes do not occur in the liver. None of these derived values for muscle and liver discloses

TABLE I. Water and Electrolyte Values in Plasma, Muscle and Liver of Control Rats and Rats Subjected to Cholinesterase Inhibitors.

Group	Plasma Determined				Muscle						
					Determined				Derived		
	(H_2O) g/l	(K^+) mEq/kg H_2O	(Na^+) mEq/kg H_2O	(Cl^-) mEq/kg H_2O	(H_2O) g	(K^+) mEq	(Na^+) mEq	(Cl^-) mEq	$(H_2O)_E$ g	$(H_2O)_C$ g	$(Na^+)_C$ mEq
K deficient											
Saline control	923	2.34	161	104	764	57.0	52.7	13.3	112	652	36.5
S.E.	3	.09	2	4	1	1.7	3.2	.3	3	1	4.3
	(6)	(5)	(6)	(6)	(3)	(6)	(5)	(5)	(3)	(3)	(3)
DFP	925	3.09	156	101	768	63.7	48.1	13.1	118	646	29.4
S.E.	1	.49	2	3	2	2.6	3.9	.8	10	5	.8
	(6)	(6)	(6)	(6)	(3)	(6)	(6)	(6)	(3)	(3)	(3)
Normal K											
Saline control	919	6.51	158	111	763	103	17.6	11.9	102	671	.5
S.E.	3	.70	4	5	—	4	.9	.5	8	18	1.7
	(4)	(4)	(3)	(4)	(1)	(3)	(4)	(4)	(3)	(3)	(3)
DFP	919	6.45	154	117	764	98.3	18.7	10.0	92	672	4.8
S.E.	2	.31	2	2	3	3.8	.5	.9	7	7	2.9
	(4)	(4)	(3)	(4)	(3)	(4)	(4)	(4)	(3)	(3)	(3)

Group	Liver						
	Determined				Derived		
	(H_2O) g	(K^+) mEq	(Na^+) mEq	(Cl^-) mEq	$(H_2O)_E$ g	$(H_2O)_C$ g	$(Na^+)_C$ mEq
K deficient							
Saline control	734	72.9	24.5	20.0	177	557	−4.0
S.E.	4	4.8	1.4	1.4	11	9	1.5
	(4)	(5)	(6)	(6)	(4)	(4)	(4)
DFP	735	80.7	23.2	20.5	172	568	.5
S.E.	4	7.0	.7	.8	21	22	1.4
	(6)	(4)	(5)	(4)	(4)	(4)	(4)
Normal K							
Saline control	719	83.4	21.8	23.1	199	532	−.3
S.E.	3	8.8	1.9	1.7	10	21	8.2
	(3)	(3)	(4)	(4)	(4)	(4)	(4)
DFP	720	85.9	19.0	20.8	185	522	−6.8
S.E.	4	3.0	1.7	1.9	10	15	1.8
	(4)	(4)	(4)	(4)	(3)	(3)	(3)

Muscle and liver data/kg fat-free whole tissue.
S.E. = Stand. error of mean. Numbers in parentheses indicate No. of observations.
Data presented for Group A1 rats only.

any difference between animals receiving and those not receiving cholinesterase inhibitors.

Histologic study. Cardiac muscle revealed myodegeneration typical of potassium deficiency(16) and renal tubular changes of uncertain origin. No differences were noted between control animals and those given Parathion and DFP.

Discussion. Alterations reported here as the result of potassium depletion are similar to those found by Heppel(17) and Yannet and Darrow(18). Changes in water and electrolyte composition of plasma, skeletal muscle, and liver could not be related to repeated administration of Parathion or DFP. Lack of evidence in these experiments that shifts of water and electrolytes occur as a specific result of cholinesterase inhibition suggests that hyperkalemia produced by acute lethal doses of anticholinesterases is unlikely to be related to changes in cellular permeability directly as a result of excessive concentrations of acetylcholine. The possibility remains that disturbance of cellular permeability may be too transient to have been encountered in these studies.

Summary. Normal rats and potassium deficient rats were injected with DFP and Para-

thion over many weeks. Electrolytes of plasma, skeletal muscle and liver of the potassium deficient rats showed the effects of potassium depletion. Studies of cholinesterase disclosed reduction in activity of this enzyme in rats receiving cholinesterase inhibitors. No effects due to cholinesterase inhibition were found upon either concentrations or distributions of water, potassium, sodium or chloride in plasma, skeletal muscle and liver.

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1. Greig, M. E., and Holland, W. C., *Arch. Biochem.*, 1949, v23, 370.

2. Holland, W. C., and Greig, M. E., *Am. J. Physiol.*, 1950, v162, 610.

3. Taylor, I. M., Weller, J. M., and Hastings, A. B., *ibid.*, 1952, v168, 658.

4. Mathias, P. J., and Sheppard, C. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 69.

5. Nachmansohn, D., *Vitamins and Hormones*, 1945, v3, 337, Academic Press, Inc. New York.

6. Rothenberg, M. A., *Biochem. et Biophys. Acta*, 1950, v4, 96.

7. Grunfest, H., and Nachmansohn, D., *Fed. Proc.*, 1950, v9, 53.

8. Holland, W. C., Dunn, C. E., and Greig, M. E., *Am. J. Physiol.*, 1952, v168, 546.

9. Fenn, W. O., Gerschman, R., Fischer, G., Lacy, J., Bailly, M. D., and Wright, J. L., *J. Gen. Physiol.*, 1951, v34, 607.

10. Freeman, G., Ludemann, H., Cornblath, M., Gold, A., Filbert, M., Udall, J., Cugell, D., and Craig, A., *Fed. Proc.*, 1954, v13, 48.

11. Orent-Keiles, E., and McCollum, E. V., *J. Biol. Chem.*, 1941, v140, 337.

12. Michel, H. O., *J. Lab. and Clin. Med.*, 1949, v34, 1564.

13. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, 1942, v143, 257.

14. Koelle, G. B., *J. Pharm. and Exp. Therap.*, 1950, v100, 158.

15. Cotlove, E., Holliday, M. A., Schwartz, R., and Wallace, W. M., *Am. J. Physiol.*, 1951, v167, 665.

16. French, J. E., *Arch. Path.*, 1952, v53, 485.

17. Heppel, L. A., *Am. J. Physiol.*, 1939, v127, 385.

18. Yannet, H., and Darrow, D. C., *J. Biol. Chem.*, 1940, v134, 721.

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Effects of Adrenalectomy on Specific Activity of Deoxyribonuclease in Rat Spleen After Total Body X-Irradiation.* (22141)

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In view of the fact that specific activity of deoxyribonuclease II (DNase II)(1) in rat spleen is more than doubled after total body x-irradiation(2), it has been of interest to determine the role of adrenal cortex in this phenomenon. Leblond and Segal(3) recorded involution of the spleen as a result of localized x-irradiation after adrenalectomy. Prolonged administration of corticotropin to rats decreases cellularity of the spleen, and therefore the amount of deoxyribonucleic acid (DNA) present(4). Reduction in tissue mass of the organ has been attributed to lymphocytolysis and the inhibition of mitosis resulting from adrenocortical steroids(5).

We have measured the DNase II activity in spleens of control, x-irradiated intact, adrenalectomized and x-irradiated-adrenalectomized rats.

Methods. Twenty-four young adult male rats of the Sprague-Dawley strain were used. They were divided into 4 groups of 6 animals each. The groups were treated as follows: Group I—control, Group II—x-irradiated-intact, Group III—adrenalectomized, Group IV—x-irradiated-adrenalectomized. All were housed in metal cages in an air conditioned room at 80°F, and fed a diet of Purina Lab-

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TABLE I. Effect of Adrenalectomy and/or 200 r of Total Body X-Irradiation on Average DNase II Activities of Rat Spleen.

Group No.	Treatment	γDNA hydrolyzed/mg tissue/hr at 37°C	Spleen wt / Body wt ratio	γDNA hydrolyzed/spleen/g body wt
I	Control (6)	14.09 ± .98*	2.35 ± .12 × 10^{-3}	32.96 ± 2.64
II	X-irradiated intact (6)	26.71 ± 1.15	1.52 ± .07 × 10^{-3}	40.83 ± 3.54
III	Adrenalectomized (6)	13.76 ± 1.08	3.26 ± .17 × 10^{-3}	44.29 ± 1.56
IV	X-irradiated and adrenalectomized (6)	28.24 ± .69	1.97 ± .11 × 10^{-3}	55.74 ± 3.76

* Stand. error.
Numbers in parentheses refer to No. of animals used.

oratory chow. They were given food and water *ad libitum*. Animals from Groups II and IV were adrenalectomized by sterile technic. Adrenalectomized animals were allowed 2 weeks for recovery, maintained on physiological saline solution rather than water for drinking. Animals from Groups II and IV were irradiated, using the following physical factors: 220 KV, 15 ma, inherent filtration only, HVL 0.25 Cu, 32 inches to center of animal, field size covering entire animal and at 35.5 r per minute in air for 5.6 minutes (200 r). Twenty-four hours later, animals from all 4 groups were weighed, stunned and killed by decapitation. Spleens were quickly dissected out, rinsed in distilled water, blotted dry on filter paper, and weighed. They were then homogenized in distilled water with an all-glass homogenizer, and homogenates diluted to give tissue concentrations of 20%. One-ml aliquots of homogenates were added to 1 ml portions of substrate solution, which contained 5 mg/ml of highly polymerized DNA† in a 0.1 M acetate buffer, pH 5, and these reaction mixtures were incubated in a water bath at 37°C for one hour. The reaction was stopped by addition of 2 ml of cold 10% trichloroacetic acid. After centrifugation and filtration, 2-ml aliquots of supernatant were reacted with Dische's diphenylamine color reagent(6). The violet color formed was measured on the Klett-Summerson photoelectric colorimeter.

Results. Results of the experiment indicate that adrenal cortex has no influence on the specific activity of DNase II/mg of rat spleen; that is, there is no significant difference between amounts of this enzyme activity per mg of tissue in control (I) and adrenalectomized (III) rats; nor is there any significant difference between x-irradiated intact (II) and x-irradiated adrenalectomized (IV) rats. However, there is a significant difference in total units of activity per spleen. Application of the "*t*" test(7) yields a P value for the change in total activity in groups I and III of $<.01$, in groups II and IV of $<.05$, in groups III and IV of $<.02$.

Results also confirm the fact that secretions of adrenal cortex have specific effects on involution of the spleen, as indicated by significant variations in spleen wt/body wt ratios in groups I and III, and groups II and IV in Table I.

Summary. Adrenalectomy has been found to have no effect on increased specific activity of rat spleen DNase II following total-body x-irradiation, but size of spleen and therefore total quantity of enzyme changed in both irradiated and unirradiated animals.

The authors wish to express their gratitude to Dr. I. Meschan, Professor of Radiology, for his kind assistance in irradiation of the animals.

† Purchased from California Foundation for Biochemical Research, Los Angeles.

1. Cunningham, L., and Laskowski, M., *Biochim. et Biophys. Acta,* 1953, v11, 590.

2. Fellas, V. M., Meschan, I., Day, P. L., and Douglass, C. D., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 231.

3. Leblond, C. P., and Segal, G., *Am. J. Roentgenol. Radium Therapy,* 1942, v47, 302.

4. Robbins, G. P., Cooper, J. A. D., and Alt, H. L., *Endocrinology,* 1955, v56, 161.

5. Dougherty, T. F., *Physiol. Rev.,* 1952, v32, 379.

6. Dische, Z., *Microchimie,* 1930, v8, 4.

7. Snedecor, G. W., *Statistical Methods,* Ames, Iowa, Iowa State College Press, 1946, p65.

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Qualitative Study of Aminoaciduria in Muscular Dystrophy and Myotonia Dystrophica.* (22142)

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Studies by Ames and Risley(1) indicated that increased amounts of amino acids were excreted in the urine of muscular dystrophy patients. However, no identifications of individual amino acids or excretion pattern studies were done. More recently Hurley and Williams(2), by means of quantitative paper chromatographic analyses, have reported higher leucine, taurine, threonine, valine and arginine levels in the urine of male patients. Glycine, alanine, serine and citrulline levels were not significantly higher than in normal urines. Urines of females were not analyzed.

In the present investigation an attempt has been made to discern the various amino acids excreted in the urine of male and female patients with muscular dystrophy and myotonia dystrophica by means of 2-dimensional filter paper chromatography. Siblings and parents of the muscular dystrophy patients were studied whenever possible.

Procedures and methods. The subjects were 43 muscular dystrophy patients ranging in age from 14 to 60 years, 9 male patients with myotonia dystrophica and 53 normal subjects. In addition, a group of 13 parents and 16 uninvolved siblings were investigated. Samples for analysis were taken from the first voided morning urine. All samples were analyzed the day of voiding or stored temporarily in the frozen state. The amino acids were determined by descending 2-dimensional filter paper chromatography, essentially by the methods of Levy and Chung(3) and Fink *et al.*(4). Aliquots of 70 microliters of urine applied to Whatman No. 1 paper in 3-microliter portions were used for analysis. The developing solvents were (1) phenol-0.1 M borate buffer, pH 8.3 (85.6:20, w/v) and (2) butanol-glacial acetic acid-water (4:1:5) — organic phase. Development in phenol was carried out at 25 ± 1°; and butanol at room temperature. Ninhydrin was used as the spray reagent(3). Because of similar R_F values, the following groups of amino acids could not be resolved: isoleucine and leucine; glutamine, citrulline and arginine; methionine and valine; and methionine sulfoxide and sarcosine. β-Aminoisobutyric and γ-amino-n-butyric acids if present would travel in the methionine-valine band. Taurine under these conditions is obscured by glycine in most instances. Further separations of these groups have not been attempted.

Results. There is a qualitative increase in the excretion of amino acids and certain related unidentified substances in muscular dystrophy patients, as shown by an increased number of spots on the urine chromatograms. The amino acids which appeared most frequently in the urine of dystrophy patients were methionine or valine, isoleucine or leu-

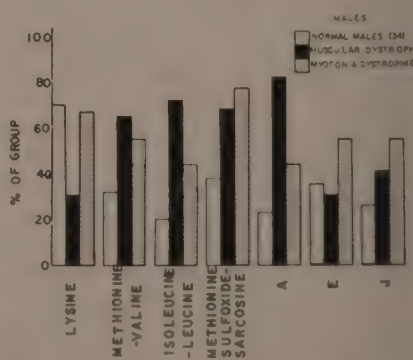

FIG. 1. Urinary amino acid excretion patterns in normal males and male patients with muscular dystrophy and myotonia dystrophica.

* This study was aided by funds from the Muscular Dystrophy Associations of America.

† This work was done during the tenure of an Established Investigatorship of the American Heart Assn.

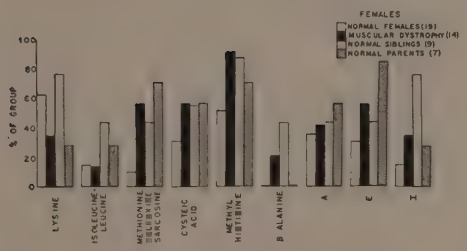

FIG. 2. Urinary amino acid excretion patterns in normal females, normal siblings, parents, and female muscular dystrophy patients.

cine, methionine sulfoxide or sarcosine, methyl histidine, cysteic acid and unidentified substances A and E (Fig. 1 and 2).‡

Nine subjects with myotonia dystrophica exhibited increased numbers of urinary amino acids. Lysine, methionine sulfoxide-sarcosine and unidentified substances E and J‡ appeared more frequently in the urine of these patients than in the urine of muscular dystrophy patients (Fig.1).

The amino acid excretion of a small group of clinically uninvolved male siblings and male parents was studied. There was a significant increase in the former group. Marked elevations of isoleucine or leucine, β-alanine, cysteic acid, and unidentified substances A and I‡ were observed. In the male parent group, methionine-valine and cystine were increased (Fig. 3).

A group of uninvolved female siblings and female parents manifested marked increases in the number of amino acids excreted (Fig. 2). The aminoaciduria was as extensive in these latter groups as in the female muscular dystrophy patients.

A relatively large number of unidentified spots were observed in the chromatograms of muscular dystrophy patients and their relatives. Spots designated A and E were most consistently present.

Discussion. The data presented indicate that a qualitative increase in urinary amino acids occurs in patients with muscular dystrophy and myotonia dystrophica and certain related normal individuals. Whether there is an associated quantitative increase in amino acid concentration in the urine of such individuals is currently under investigation.

The increased numbers of amino acids observed in the urine of normal siblings and parents of muscular dystrophy patients suggests the possibility of an inherited metabolic abnormality. The particularly large numbers of amino acids found in the urine of the normal female sibling and the maternal parent are striking evidence in favor of this thesis. Furthermore, genetic studies seem to indicate maternal transmission of the disease(6).

The presence of the relatively large group of unidentified ninhydrin staining substances, found in the urine of dystrophy patients and related individuals, is of interest. These components may be unidentified amino acids or peptides. It seems likely that these are normal constituents of urine, excreted at much lower levels in normal individuals since the urines of controls often contain these components. Boulanger, Biserte and Courtot(5) have described peptides in normal urines, some of which may be identical to those observed here. Two spots, A and E, are most consistently present in dystrophic individuals and their immediate relatives. The nature of these unknown substances is currently under study.

Summary. 1. An increase in the number of urinary amino acids and certain related unidentified substances was observed in muscular dystrophy and myotonia dystrophica patients. 2. Increased numbers of amino acids were found in the urine of normal sib-

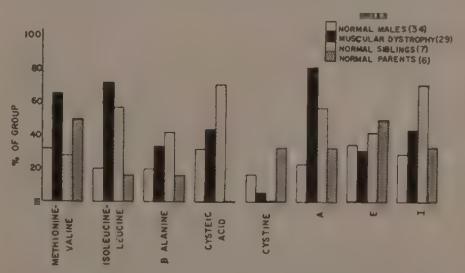

FIG. 3. Urinary amino acid excretion patterns in normal males, normal siblings, parents, and male muscular dystrophy patients.

‡ The R_F values of these unknown ninhydrin-reacting substances in solvents 1 and 2 respectively are— A: 0.75, 0.29; E: 0.50, 0.29; I: 0.85, 0.17; and J: 0.85, 0.11.

lings and the maternal parents of muscular dystrophy patients.

The authors are indebted to Drs. Franz K. Bauer and Augustus S. Rose for their advice and encouragement throughout the course of this study.

1. Ames, S. R., and Risley, H. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 131.

2. Hurley, K. E., and Williams, R. J., *Arch. Biochem. Biophys.*, 1955, v54, 384.

3. Levy, A. L., and Chung, D., *Anal. Chem.*, 1953, v25, 396.

4. Fink, K., Henderson, R. B., and Fink, R. M., *J. Biol. Chem.*, 1952, v197, 441.

5. Boulanger, P., Biserte, G., and Courtot, F., *Bull. Soc. Chim. Biol.*, 1952, v34, 366.

6. Stephens, F. E., *Am. J. Med.*, 1953, v15, 558.

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Effect of a High Fat Fructose and Casein Diet on Diabetic Cataracts.* (22143)

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Two or 3 months after the chemical induction of diabetes in rats, the lenses of the animals become opaque(1,2). The rate at which these cataracts develop is directly related to the height of the blood sugar(2). Decreasing the hyperglycemia by partial starvation(3) or the administration of phlorizin(4) delays or prevents cataracts to the extent that the blood sugar is lowered. These results suggest that hyperglycemia may play a direct role in the production of cataracts. If the high blood sugar level acts directly on the lens to produce cataracts, then the amount of sugar reaching the eye by way of the bloodstream should be of importance. Altering the blood supply by unilateral carotid ligation influences the development of galactose cataracts but does not affect the development of diabetic cataracts(5). This finding raises a question regarding the direct role of hyperglycemia and leads to the suggestion that the prevention of cataracts might be due to the provision of an alternate energy supply by the ketosis that accompanies phlorizin administration or starvation. Diets containing large amounts of fat or protein provide non-carbohydrate substrates for energy and delay or prevent cataracts(1,6). However, these diets also lower the blood sugar and an interpretation of the results is difficult. Experiments in this laboratory indicate that a high-fat diet containing fructose rather than glucose, can be fed to diabetic rats without producing a depression in the blood sugar level. The use of this diet makes it possible to determine the effect of the dietary constituents in the prevention of cataracts. This paper reports the results obtained with a diet containing large amounts of fructose, fat and protein.

Methods. Male Sprague-Dawley rats weighing 76-173 g were made diabetic by the intravenous injection of 40 mg per kilo of alloxan monohydrate. The animals were maintained with unlimited amounts of water and ground dog chow. They were weighed and the blood sugars(7) determined once or twice each week. At the end of the first four weeks the blood sugar determinations for each rat were averaged. Those animals with blood sugar levels greater than 350 mg per 100 cc of blood were retained in the experiment and divided into two groups. The control group was maintained on the same diet as had been used in the first 4-week period. The experimental group was placed on an unlimited amount of the special diet. The latter diet consisted of 50% fructose, 25% corn oil and

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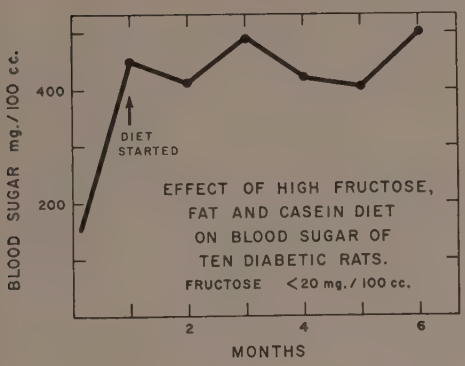

FIG. 1.

25% casein, fortified with an adequate amount of minerals and vitamins. Blood sugars were determined at least once each week and averaged for each 4 week period. During the next 4 months, 4 of the animals on the special diet had average blood sugars which fell below 350 mg per 100 cc of blood on one or more occasions. These animals were dropped from the experiment. Ten animals on the special diet and 10 receiving the chow diet maintained blood sugars consistently above 350 mg per 100 cc.

Results. The effectiveness of fructose in maintaining a high blood sugar level, in spite of a large amount of fat in the diet, is indicated by the blood sugar averages as depicted in Fig. 1. The blood sugar values represent the total amount of glucose and fructose in the blood. Separate determinations for fructose(8) on the same blood samples indicated that the fructose level was 10-20 mg per 100 cc.

The eyes of the rats were examined daily for cataracts as previously described(2). The control group developed cataracts between 59 and 120 days after the induction of diabetes with an average time of 73 days and a standard deviation of 18 days. The rats on the special diet were observed for a period equal to twice this average time or 150 days. During this period 2 of the 10 rats developed cataracts at 86 and 99 days. These cataracts occurred 2 and 3 weeks after a 3-day lapse in the special diet during which time the animals were fed a ground chow diet. The other 8 rats did not have cataracts at 150 days (Fig. 2).

Rats placed on the special diet showed a marked change in their growth rate. Before being placed on the special diet, the animals grew at the expected rate for diabetic rats having a blood sugar of 450-500 mg per 100 cc. After the initiation of the special diet, however, the growth rate was accelerated and comparable to that of diabetic rats with blood sugars between 250-300 mg per 100 cc. This was true in spite of the fact that the blood sugar of these animals was maintained at the higher level (Fig. 3).

Discussion. There are 3 effects of the high fructose, fat and casein diet which are worthy of further comment. First, it maintains a high blood sugar level. Second, it prevents the development of cataracts and third, it accelerates growth.

When a high fat diet containing sucrose is fed to diabetic rats, there is no glycosuria and the growth of the rats is stopped(1). In contrast, a high fat diet containing fructose results in a blood sugar comparable with that of a glucose low fat diet and the animals gain weight. It is possible that a high content of fat in the diet interferes with the intestinal absorption of glucose and not of fructose. Further studies are required to prove this point. The observation that there is very little fructose in the blood of a fructose fed animal is consistent with the fact that fructose is rapidly converted into glucose(9).

The fact that a high fructose, fat and casein diet prevents the development of cataracts definitely rules out hyperglycemia as a direct mediator in the production of cataracts and demonstrates that diabetic cataracts can

EFFECT OF HIGH FRUCTOSE, FAT AND CASEIN DIET ON DEVELOPMENT OF DIABETIC CATARACTS.

DIET	NO. RATS	AVE. WT. gm.	BLOOD SUGAR mg./100 cc. CONTROL	BLOOD SUGAR mg./100 cc. ON DIET	PER CENT WITH CATARACTS AT 150 DAYS (50, 100)
SPECIAL	10	125	450	448	P <0.001
CHOW	10	129	490	475	

FIG. 2.

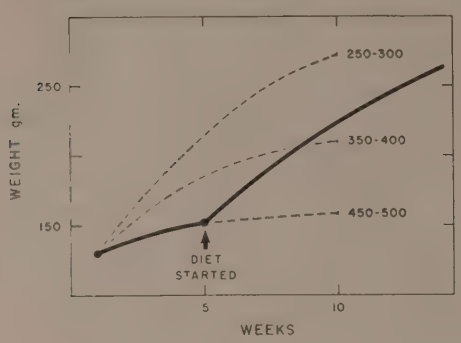

FIG. 3.

be prevented without insulin if energy sources other than glucose are made available. The results are consistent with the suggestion that diabetic cataracts are the result of an inability to obtain adequate energy by the utilization of glucose in the absence of insulin.

The growth rate of rats on a high fructose, fat and casein diet is accelerated. This indicates that tissues, other than lens, are benefited by a diet which provides substrates that do not require insulin for utilization. Rats on a high fat diet containing sucrose show no gain in weight over a period of three months (1). Thus, the presence of fructose provides an advantage. This may be due to the production of a high blood glucose level, and hence, a greater gradient for the penetration of glucose into the cells, or it may be due to the penetration of small amounts of fructose into the cell. It is doubtful whether this improvement in the distribution of carbohydrate is in itself responsible for the improved growth rate. Diets containing fructose without fat(9) and high blood levels of glucose in the absence of fat do not accelerate the growth of diabetic rats. It is more likely that the improved distribution of carbohydrate increases the efficiency with which fat is utilized by the cell. This might be brought about by increasing the availability of oxalacetic acid which is essential for the complete metabolism of fat.

Summary. A diet consisting of 50% fructose, 25% corn oil and 25% casein and fortified with vitamins and minerals was fed to diabetic rats with blood sugar levels over 350 mg per 100 cc of blood. On this diet the blood sugar was maintained at a high level, cataracts were prevented and growth rate accelerated. The beneficial effects of this diet suggest that cataracts and the failure in growth of diabetic rats are the result of a deficit of energy resulting from inadequate glucose utilization in the absence of insulin.

1. Charalampous, F. C., and Hegsted, D. M., *Am. J. Physiol.*, 1950, v161, 540.
2. Patterson, J. W., *ibid.*, 1951, v165, 61.
3. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 395.
4. ———, *Am. J. Physiol.*, 1952, v172, 77.
5. ———, *ibid.*, 1955, v180, 495.
6. Rodriguez, R. R., and Krehl, W. A., *Yale J. Biol. and Med.*, 1951, v24, 103.
7. Folin, D., and Malmros, H., *J. Biol. Chem.*, 1929, v83, 115.
8. Higashi, A., and Peters, L., *J. Lab. Clin. Med*, 1950, v35, 475.
9. Sarrett, H. P., and Snipper, L. P., *J. Nutr.*, 1954, v52, 525.

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Growth of Spherules of *Coccidioides immitis* in a Chemically Defined Liquid Medium. (22144)

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The dimorphic fungus, *Coccidioides immitis,* undergoes a completely different type of growth *in vivo* from that *in vitro.* The parasitic form, or spherule, appears in animal tissue as a non-budding, spherical structure 20-80 μ in diameter with a thick, refractile, double wall, and is filled with numerous small endospores 2-5 μ in diameter. Reproduction takes place by rupture of the spherule wall, freeing the endospores, which increase in size and develop into typical large endospore-filled spherules. The saprophytic form appears in laboratory media as a mycelium with branching, septate hyphae which break up into numerous thick-walled, rectangular, ellipsoidal, or spherical arthrospores 2-4 μ in diameter. The arthrospores germinate and repeat the cycle. Observation of the parasitic phase in solid or semi-solid complex medium has been reported by several authors. MacNeal and Taylor(1) observed multiplication of spherules in infected pus when added to ascitic fluid or gelatinized horse serum containing sterile kidney slices. Lack(2) noted development of spherules from chlamydospores inoculated into Hall tubes containing glucose broth and partially coagulated egg albumin. Baker and Mrak(3) observed the development of spherules in a variety of solid media after 45 to 60 days, when active growth had ceased due to drying of the medium. Conant and Vogel(4) observed spherules on Sabouraud's dextrose slants following exposure of the cultures to Tween 80 (polyoxyethylene (20) sorbitanmonooleate). Burke (5) reported the appearance of spherules usually after 4 to 10 weeks, but occasionally after 11 days in a partially defined solid medium containing coconut milk.

Experiments in this laboratory resulted in the production of spherules in a liquid synthetic medium, following the observation of the parasitic phase in cultures grown in the presence of lithium hydroxide and tamol "N" (an arryl sulfonic acid). The following presentation is devoted to a description of the medium, the development of the spherules, and the morphology of the cultures.

Methods and materials. 1. Inoculum used was a culture of *C. immitis,* strain M11, grown for 28 days at 34°C on a reciprocating shaking machine operating through a 4½ inch stroke at 96 strokes/minute. Growth medium for the inoculum was a glucose, acetate, inorganic salts medium, Basal #2, Roessler *et al.*(6) supplemented with 4 ppm Zn^{++} as $ZnSO_4$. The inoculum (0.1 ml) was approximately 10 x 10^6 viable arthrospores. 2. The experimental medium was a 5-fold dilution of the inoculum medium, containing .022 M glucose, .016 M ammonium acetate, .003 M each dipotassium and monopotassium phosphate, .0016 M magnesium sulfate, .0000124 M zinc sulfate and distilled water,

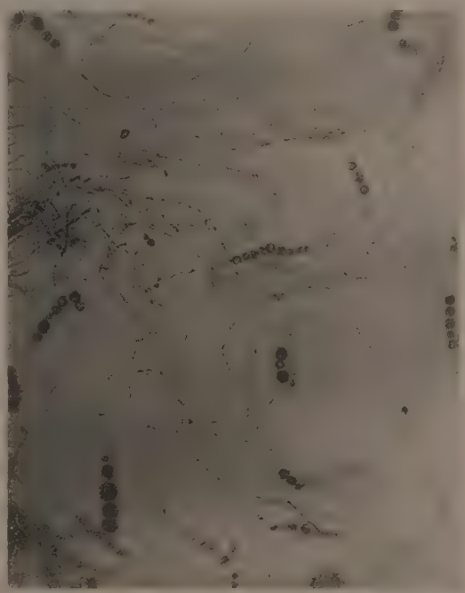

FIG. 1. Representative field of *C. immitis* culture showing growth of spherules, 200 ×.

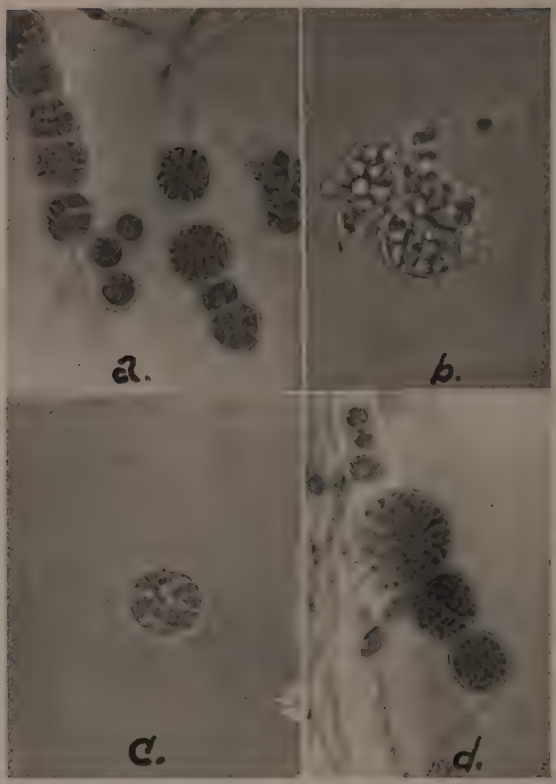

FIG. 2. (a) Spherules in various stages of development, 450 ×. (b) Two mature spherules with ruptured walls. Endospores are being extruded into the surrounding medium, 650 ×. (c) Young spherule showing developing cross-walls, 450 ×. (d) Chain of mature spherules. Note apparent rupture of wall of largest spherule and start of endospore release, 450 ×.

with final pH 6.6. This was dispensed into 250 ml Erlenmeyer flasks, 50 ml per flask, and autoclaved 15 minutes at 121.5°C. The flask closures were constructed from modified thistle tubes filled with gauze covered cotton and inserted through rubber stoppers. 3. Experimental cultures were incubated with shaking at 34°C and examined microscopically at 24-hour intervals. 4. *Evaluation.* Visual estimation of growth was made with unaided eye. Viable plate counts were made with a 1% peptone, 2% glucose, 0.1% autolysed yeast, 2% agar medium, with incubation at 34°C for 48 hours. Microscopic examinations were made on lactophenol-blue wet mounts. For photomicrographic purposes the edges of the cover slips on the wet mounts were sealed with Duco cement.

Results. (1) *Development of the parasitic form.* Spherules appeared after 72 hours incubation, following initial mycelial growth, with maximum production occurring between 96 and 120 hours. They occurred at terminal and intercalary locations of the hyphae similar to typical chlamydospore development on solid medium, and developed from chains of arthrospores, the typical spore-form of the *in vitro* type of growth in liquid medium. Upon continued incubation there appeared to be simultaneous germination of arthrospores into hyphae which produced both new arthrospores and spherules, and rupturing of spherule walls with the release of endospores which developed into new, mature, endospore-filled spherules. The parasitic form of growth appeared to comprise 50 per cent or more of the

total growth of the cultures.

(2) *Spherule morphology.* The spherules were morphologically similar to those occurring in animal tissue. They occurred as spherical or ovoid structures, 3 to 80 μ in diameter with double refractile walls, and contained varying numbers of endospores, depending on the size and stage of development of the spherules. The diameter of the endospores varied from 1 to 5 μ with the majority appearing to be one-tenth to one-fifth the volume of mature arthrospores which are normally 3 to 5 microns in diameter. The accompanying figures show representative fields of view and detailed morphology of a typical culture of *C. immitis* grown under the conditions described.

(3) *Other strains.* Although the majority of this work was accomplished with *C. immitis,* strain M11, a rodent isolate from Arizona contributed by Dr. C. E. Smith of Berkeley, Calif., 38 other strains isolated from humans and animals have been tested under identical conditions. Sixty per cent of these developed spherules in the experimental medium, but to a lesser extent than strain M11.

4. *Growth measurements.* The total growth of spherule cultures (15 x 10^6 per ml) in 5 fold diluted medium, as measured by viable plate counts, was approximately one-tenth that of arthrospore cultures (130 x 10^6 per ml) grown in the inoculum medium.

Discussion. Conditions for the growth and maintenance of the parasitic form of *C. immitis* in a chemically defined liquid medium are not yet fully understood although physical, chemical, and nutritional studies should throw further light on this subject. There seems to be a critical stage during the early development of the cultures at which time the environment exerts its greatest effect in stimulating production of spherules. Harvesting the spherules must be done not later than the fourth or fifth day of incubation as a gradual decrease in numbers takes place thereafter, accompanied by a slow reversion of the cultures to the saprophytic phase. A possible explanation might be a selective change of environment involving the exhaustion of nutritional factors or the accumulation of metabolic products inhibiting growth of the parasitic form.

The use of a chemically defined liquid medium for production of spherules would materially benefit physical, chemical, and metabolic studies of the parasitic phase of the organism. It would also permit large scale production of antigenic material, free from foreign protein matter.

Summary. A method for growth of the parasitic phase (spherules) of *Coccidioides immitis* in a chemically defined liquid medium is described. A description of the development and morphology of the structures is included.

1. MacNeal, W. J., and Taylor, R. M., *J. Med. Res.,* 1914, v30, 261.

2. Lack, A. R., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 907.

3. Baker, E. E., and Mrak, E. M., *Am. J. Trop. Med.,* 1941, v21, 589.

4. Conant, N. F., and Vogel, R. A., *Mycologia,* 1954, v46, 157.

5. Burk, R. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 332.

6. Roessler, W. G., Herbst, E. J., McCullough, W. G., Mills, R. C., and Brewer, C. R., *J. Inf. Dis.,* 1946, v79, 12.

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Dissecting Aortic Aneurysm in Hamsters Treated with Cortisone Acetate. (22145)

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Dissecting aortic aneurysm has been produced in dogs by thermal coagulation of the aorta(1), and in rats by diets containing meal of the seeds of *Lathyrus odoratus*(2-4). No report has been found of the production of this aortic lesion in any other laboratory animals. During experiments in which hamsters were treated with cortisone to accomplish heterologous transplantation of human fetal lung tissue, many animals died with massive intrathoracic or intraperitoneal hemorrhage. Histologic examination of mediastinal and retroperitoneal tissues revealed that the hemorrhage was usually due to rupture of a dissecting aneurysm of the aorta.

Materials and methods. Seventy-seven golden hamsters 1 to 3 months of age, from the colony at the National Institutes of Health (NIH) were treated with cortisone acetate and inoculated with human fetal tissue. Fifteen additional animals were treated with cortisone but received no transplants. The fetal lung tissue was cut into small fragments and implanted with an 18 gauge trocar, or was minced with small quantity (usually less than 1 ml) of physiological saline and injected by using 1 ml tuberculin syringe and 20 gauge needle. Inoculations were made into submucosa of the cheek pouch of hamsters anesthetized with veterinary nembutal, 0.11 ml/100 g body weight. Cortisone acetate (Cortone,® Merck) was injected subcutaneously into the posterior cervical region in doses of 3 mg at the time of transplantation of human tissues, and 1 mg given twice a week thereafter for remainder of the life of the animal. Hamsters were kept in large plastic cages in air-conditionsd rooms at approximately 78°F. The diet consisted of Purina Laboratory Chow and water *ad libitum,* supplemented with kale, carrots, and apples 3 times a week. Hamsters were examined daily, and dead animals necropsied immediately. Six hamsters were killed when they appeared moribund. The other 71 were found dead, but had not appeared moribund on the preceding day. At first, only the cheek pouch area was examined histologically, since the state of heterologous embryonic tissue was our primary concern. When it was apparent, however, that many animals had died with massive thoracic or abdominal hemorrhage, an attempt was made to determine the origin of the hemorrhage. Entire thoracic contents were then removed at necropsy. Lungs were removed, and remainder of the specimen including thoracic aorta was fixed in Helly's formal-Zenker fluid. The abdominal aorta was also prepared for histological study. When hardened the tissues were cut transversely into 15 to 20 blocks. After tissues were imbedded in paraffin, sections were cut at 6 μ and stained with hematoxylin and eosin routinely. A variety of histochemical technics has also been used, and these will form the basis for a later report.

Results. Massive hemorrhage was the cause of death of 51 of 77 NIH hamsters dying 26 to 162 days after initiation of cortisone therapy and transplantation of human fetal tissue. Of these 51 animals, 37 had hemothorax and 14 had hemoperitoneum. The aorta of 26 of these animals was examined microscopically, and dissecting aneurysm was demonstrated in 23. No inflammatory lesions were found. However, in sections from 3 other animals, necrotizing arteritis of the aorta was observed; this necrosis was so extensive that we were unable to determine whether dissection of the aorta had occurred. In addition, dissecting aneurysm was found histologically in 2 animals in which mediastinal hemorrhage had not taken place. Fourteen hamsters, 2 months old, obtained directly from Lakeview Hamster Colony in New Jersey were treated with

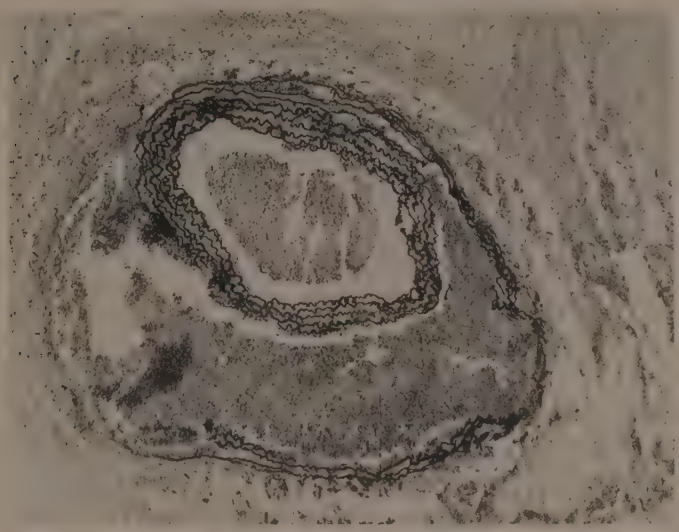

FIG. 1. Thoracic aorta of a cortisone treated hamster showing separation of elastic fibers by hemorrhage. Orcein. 60 ×.

cortisone acetate following implantation of pieces of human fetal lung tissue by the same technics as were applied to the NIH hamsters. Six of these animals died, all with hemothorax or hemoperitoneum. A control experiment to test the effect of cortisone alone is in progress. Five Lakeview and 10 NIH hamsters have been treated with cortisone acetate using the same dosage schedule, but without tissue transplantation. After 80 days 2 Lakeview and 4 NIH hamsters died with hemothorax or hemoperitoneum, and dissecting aneurysm has been demonstrated histologically in all.

Histologically, the aneurysmal dissection of aortic wall appeared to originate at a point lying just above aortic valve. The lesion consisted of separation of layers of the media of the aorta by extravasated blood (Fig. 1). In the sections of aorta from the 2 animals without massive hemorrhage, there was hyalinization of areas of the outer media adjacent to the aneurysmal vascular lumen. In animals with massive hemorrhage, medial necrosis was no longer evident histologically. The structural continuity of the blood-filled intramedial spaces with lumen of the vessel on the one hand or with perivascular tissue on the other, could be demonstrated in some sections. Most of the aneurysms appeared to have developed acutely, since there was little evidence of inflammatory reaction in areas of dissection or hemorrhage.

Discussion. The factors involved in the degenerative lesion that eventuates in weakening of the tunica media and development of these dissecting aneurysms in hamsters, are not clear from these experiments. We have no evidence to suggest that this lesion is related to lathyrism. The diet contained no pea meal, although Purina Laboratory Chow does include legumes alfalfa and soybean. Gross examination has not shown striking abnormalities of bone which have been described in lathyrism(4). The diet has been fed to nearly a thousand other hamsters in our laboratory over long periods of time, and none have shown evidence of aneurysm. Water extraction of Purina Chow according to the method of Dupuy and Lee(5) has yielded an amorphous yellowish substance. The relationship, if any, between this material and the crystals isolated from *Lathyrus pusilis* awaits completion of biological tests now in progress. The possibility of a synergism between cortisone and some dietary factor is still to be considered.

No report of aneurysm in patients treated

with cortisone or corticotrophin has been found, although necrotizing arteritis was described recently following self-administered cortisone overdosage in an arthritic patient (6). Other investigators have found aortas of cortisone-treated children to have a high content of lipids(7). Degeneration of the tunica media of the ascending aorta without hemothorax has been observed in this laboratory in two mice bearing a subcutaneous transplant of a functional adrenal cortical carcinoma(8).

Summary. Massive thoracic or abdominal hemorrhage has been found in 51 of 77 hamsters treated for 26 to 162 days with cortisone acetate. All of these animals bore implants of human fetal tissue. Histological sections of 26 of these animals demonstrated dissecting aortic aneurysm in 23 and necrotizing arteritis in 3 animals. The aorta of the other 25 animals with gross hemorrhage was not examined histologically. Six non-transplant-bearing hamsters treated with cortisone died with microscopically proved dissecting aortic aneurysm.

1. Schlichter, J. G., *Arch. Path.*, 1946, v42, 182.
2. Ponseti, I. V., and Baird, W. A., *Am. J. Path.*, 1952, v28, 1059.
3. Bachhuber, T. E., and Lalich, J. J., *Science*, 1954, v120, 712.
4. Ponseti, I. V., *J. Bone Surg.*, 1954, v36-A, 1031.
5. Dupuy, H. P., and Lee, J. G., *J. Am. Pharm. Assn.*, 1954, v43, 61.
6. Finck, P. A., *A.M.A. Arch. Path.*, 1955, v60, 374.
7. Etheride, E. M., and Hoch-Ligeti, C., *Am. J. Path.*, 1952, v28, 315.
8. Dunn, T. B., unpublished observations.

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Inhibition of Calcification *in vitro* by Luteocobalti Chloride.* (22146)

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There is evidence that chondroitin sulfate or its protein complex may play a role in the process of calcification. Rubin and Howard (1) found an increase in metachromatic staining in regions where active calcification is occurring, which they attribute to increase in concentration of chondroitin sulfate or a change in its degree of polymerization. Neuman(2) showed a correlation between cation binding capacity of cartilage and its sulfate content. Miller, Waldman and McLean(3) found that basic dyes inhibit calcification *in vitro* and that this effect can be reversed in the presence of calcium and phosphate. Finally, it has been demonstrated that protamine, which can precipitate chondroitin sulfate from solution, can cause inhibition of calcification *in vitro*(4,5). Like protamine, the complex luteocobalti cation can precipitate chondroitin sulfate as shown by Matthews and Dorfman(6). It will also precipitate mucoprotein of cartilage(7), as well as ribonucleic acid, heparin and sodium polymetaphosphate, from solutions of low ionic strength and at a pH above 3.5(6). Under similar conditions hyaluronate, neutral polysaccharides and human serum proteins fail to precipitate.

In view of this property of acting as a rather selective precipitant, it was decided to study the effect of luteocobalti chloride on calcification *in vitro*. It was also considered that the orange-yellow color of the cation might make it possible to detect its presence at calcification sites.

Methods. Calcification *in vitro* was studied by a simplification of the method described by Sobel, Nobel and Hanok(8). Twenty-one-day-old weanling rats were

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raised on a standard rachitogenic diet (U.S.P. Rachitogenic Diet No. 2). When they were 5 to 6 weeks old, they were sacrificed, the tibia was dissected out and sliced longitudinally. These tibial sections were placed in calcifying solution (described below) and incubated at 37°C for 16 to 20 hours. Sections were then washed with water, placed in 2% aqueous silver nitrate and exposed to the light of incandescent lamp for about 10 minutes. Calcification manifests itself as a dark deposit on the surface of the epiphyseal plate. It is measured by extent of this deposit, using numbers from 0 to 4, to designate width of surface of the plate covered and by zero or 1 to 4 plus signs, enclosed in parenthesis, to designate length of plate covered. Range of values extends from 0 (0) to 4 ($^{++++}$). The action of luteocobalti chloride was studied in 3 ways; (1) incorporation of inhibitor into the calcifying solution itself, (2) prior exposure of sections to inhibitor in the basal salt solution, followed by washing with water and incubation in calcifying solution, and (3) prior exposure of sections to inhibitor in the presence of calcium chloride, followed by washing and incubation in the calcifying solution. The calcifying solution contained the following components: sodium chloride (0.07 M), potassium chloride (0.005 M), sodium bicarbonate (0.022 M), calcium chloride (0.0025 M; 10 mg% calcium), and a combination of NaH_2PO_4 plus Na_2HPO_4 at a molarity of 0.00161 corresponding to 5 mg% phosphorus. The pH of the solution was adjusted to 7.3 with carbon dioxide. The basal salt solution consisted of the following: sodium chloride (0.07 M), potassium chloride (0.005 M), and sodium bicarbonate (0.022 M). The solution was adjusted to a pH of 7.3 with CO_2.

TABLE I. Inhibition of Calcification *In Vitro* by Addition of Luteocobalti Chloride to Calcifying Solution.

Conc. of LCC (normality)	No. of experiments	Degree of calcification (avg)
.0	9	1½(++++)
.0056	3	1 (+)
.009	2	1 (++)
.019	2	0 (0)
.028	4	0 (0)
.056	2	0 (0)
.112	7	0 (0)

LCC = Luteocobalti chloride.

TABLE II. Inhibition of Calcification *In Vitro* by Prior Exposure to Luteocobalti Chloride.

Conc. of LCC (normality)	No. of exps.	Degree of calcification (avg)
.0	5	1(++++)
.0–(B.S.)	5	1 (+++)
.0011	2	½ (+++)
.0022	2	½ (+)
.003	2	0 (0)
.0056	4	0(0), 0(0), 0(0), 1(++++)
.011	4	0 (0)
.056	3	0 (0)
.112	3	0 (0)

B.S. = Basal salt solution.

Results. The results in Table I were obtained by addition of luteocobalti chloride to the calcifying solution in concentrations indicated. Inhibition began in the concentration range 8.96×10^{-3} to 1.9×10^{-2} N luteocobalti chloride. There was a deposition of cobalt ion on the surface of the epiphyseal plate increasing with concentration of luteocobalti chloride employed.

The results in Table II were obtained by prior exposure of tibial sections to luteocobalti chloride in the presence of basal salt solution. The system was shaken intermittently for 2¼ hours. Then the tibial sections were washed with water and placed in calcifying medium for 16 to 20 hours. The sample designated as zero normality of luteocobalti chloride is a control which received no prior treatment; B.S. indicates a control that received prior treatment with basal salt solution. Inhibition of calcification begins in the range 2.2×10^{-3} N to 3.4×10^{-3} N, if one ignores the single case of inhibition found at 5.6×10^{-3} N.

It has been demonstrated by Sobel, Nobel and Hanok(8) that inhibition of calcification *in vitro* by prior shaking with neutral salts may be reversed by subsequent shaking with calcium chloride. In view of this it was decided to determine the effect of calcium chloride on inhibitory action of luteocobalti chloride. Table III shows results of prior exposure of tibial sections to luteocobalti chloride in the presence of calcium chloride for 1½ hours, followed by their exposure to calcifying solution.

TABLE III. Effect on Calcification of Prior Treatment with Both Luteocobalti Chloride and Calcium Chloride.

Conc. of LCC (normality)	Conc. of $CaCl_2$ (normality)	No. of samples	Degree of calcification (avg)
.00	.0	7	1½ (+++)
.00–($CaCl_2$)	.5	6	1½(++++)
.0056	.5	4	2 (++++)
.011	.5	5	1½(++++)
.0179	.5	4	1½(++++)
.028	.5	5	1½(++++)
.056	.5	4	½ (+++)
.112	.5	6	1 (+++)
.056	.001	6	1 (++++)
.056	.05	7	1 (+++)
.056	.10	5	½(++++)

There is no complete inhibition of calcification. Calcium chloride appears to exert a protective effect on the calcifying system. The amount of cobalt deposited, indicated by depth of yellow color at the surface of epiphyseal plate, depended on the ratio of luteocobalti chloride to calcium chloride. The greater the concentration of the calcium chloride, the less the deposit of the cobalt salt. In this connection it was observed that precipitates, formed from luteocobalti chloride and chondroitin sulfate in aqueous solution, readily dissolved in the presence of calcium chloride.

The most probable binding site of luteocobalti chloride at cartilage surface is either the chondroitin sulfate or its mucoprotein. In view of the possibility of mucoprotein involvement, it was decided to determine the effect of trypsin on the binding of luteocobalti chloride by tibial sections. In these experiments tibial sections were exposed to a solution of Tryptar (lyophilized crystalline trypsin, Armour) at concentration of 10 mg/ml in water for the times indicated in Table IV. The sections were then washed and exposed to luteocobalti chloride (.2%) for one hour. Evaluation of cobalt deposit was similar to that used in evaluation of calcification. However, due to the nature of the cobalt color, evaluation tends to be more subjective. The final value of the cobalt deposit was obtained by multiplying the number for length by the number for width of deposit. The results indicate a complete loss of capacity to bind luteocobalti chloride on exposure to trypsin for 75 minutes.

TABLE IV. Effect of Trypsin on Capacity of Epiphyseal Sections to Bind Luteocobalti Chloride.

Exposure time to trypsin (min.)	No. of sections	LCC deposit	Final value of LCC deposit
Control	1	3(++++)	12
15	1	2(++++)	8
37	1	1(++++)	4
45	2	0 (0)	0
50	1	½(++++)	2
60	1	½ (++)	1
75	2	0 (0)	0
90	2	0 (0)	0

Discussion. The work reported here supports the view that chondroitin sulfate or a mucoprotein containing it may be one component of calcifying systems. The luteocobalti cation, to the extent that it is a relatively specific precipitant for chondroitin sulfate, may be more useful than other cations for studying reversible inhibition of calcification. The fact that nasal cartilage does not calcify *in vitro* under the test conditions, shows that a high content of chondroitin sulfate or its mucoprotein is not a sufficient condition to cause calcification.

Summary. 1. Luteocobalti chloride inhibits calcification *in vitro* when tibial sections are exposed to its action, either prior to their exposure to the calcifying solution, or concomitant therewith. The inhibition is antagonized by calcium ions. 2. That the luteocobalti cation deposits on the surface of the epiphyseal plate can be seen because of its color. This deposit is prevented by calcium ions. 3. The capacity of tibial sections to bind luteocobalti chloride is destroyed by their preliminary exposure to trypsin.

1. Rubin, P. S., and Howard, J. E., *Trans. of Macy Conf. on Metabolic Interrelations*, 1950, v2, 155.

2. Neuman, W. F., Boyd, E. S., and Feldman, I., *ibid.*, 1952, v4, 100.

3. Miller, Z. B., Waldman, J., and McLean, F. C., *J. Exp. Med.*, 1952, v95, 497.

4. Harris, A. F., *The Biochemistry of Calcification*, Thesis for M.S. Degree in Chemistry, Polytechnic Inst. of Brooklyn, N. Y., 1952.

5. Sobel, A. E., and Burger, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 7.

6. Mathews, M. B., and Dorfman, A., *Arch. Biochem.*, 1953, v42, 41.

7. Shatton, J., and Schubert, M., *J. Biol. Chem.*, 1954, v211, 565.

8. Sobel, A. E., Nobel, S., and Hanok, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 68.

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Absorption in Dogs Following Exclusion of Bile and Pancreatic Juice Using I^{131} Labeled Fat. (22147)

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(Introduced by Ivan W. Brown, Jr.)

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For the past year this laboratory has utilized I^{131} labeled protein† (albumin) and fat† (glycerol trioleate) in studying absorption and digestion in various disease states, *i.e.* pancreatic disorders, ulcerative colitis, small intestinal disorders such as sprue and Whipple's disease, and in post gastrectomy patients. In comparative studies using radioactive iodinated serum albumin and radioactive iodinated glycerol trioleate in dogs subjected to total pancreatectomy, there was a greater abnormality in digestion and absorption of the radioactive fat than in digestion and absorption of I^{131} labeled albumin as measured by percentage of radioactivity found in blood following the test meal containing these substances(1).

The present study was done to further evaluate the effect of exclusion of bile and pancreatic juice on the pattern of fat digestion and absorption by use of a fat test meal containing I^{131} labeled glycerol trioleate.

Materials and methods. Two types of fat meal were prepared, each identical except that one was emulsified in a Waring blender and contained 2-3 ml of polyoxyethylene sorbitan mono-oleate (Tween 80). The constituents of the meal were: ½ ml of peanut oil‡/kg body weight of the animal, 10 μc of I^{131} labeled glycerol trioleate, the total mixed with equal volume of water. For 3 days prior to the test meal the animals were given .99 g tablet of potassium iodide daily to saturate the thyroid with iodine. All animals were fasted for 12 hours prior to the test. A rubber Levine tube was passed into the stomach of the unanesthetized animal and the test meal given via the stomach tube. 2 ml of venous blood was then drawn at 1, 2, 3, 4, 5, and 6 hours following the test meal into the stomach. Each sample of blood was then assayed for radioactivity in a calibrated sodium iodide thallium activated scintillation crystal well counter. Using measured radioactivity of the 2 ml sample and the calculated blood volume of animal (assumed to be 7.5% of body weight), the total radioactive content of blood was calculated and expressed as percentage of total ingested radio-iodine. From these data a curve was constructed showing the relationship of radioactivity in blood as a function of time. To establish a "normal" pattern of radioactivity of blood following a fat meal of peanut oil containing I^{131} labeled glycerol trioleate, 39 tests were done on 24 normal dogs. In 29 of these tests peanut oil and glycerol trioleate constituting the test meal were emulsified. Ten additional tests were done on 10 dogs in which the constituents were not emulsified. Ten dogs were then subjected to total pancreatectomy under sodium amytal anesthesia, following which they were given a diet as suggested by Markowitz (2) with daily injection of insulin. Two-three weeks following operation these animals

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† Obtained from Abbott Laboratories, Chicago, under A.E.C. authorization.

‡ Obtained from Planters Nut and Chocolate Co., Wilkes-Barre, Pa.

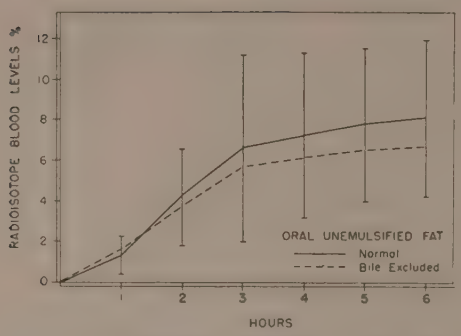

FIG. 1. Graph showing comparison in mean curve of blood isotope percent in 10 normal and 10 bile duct occluded dogs using an I^{131} labeled unemulsified fat test meal.

were tested with the peanut oil and I^{131} labeled trioleate emulsified meal. Fourteen additional dogs had ligation of their common bile duct and two weeks later were tested with the emulsified fat meal. Ten of these duct ligated animals were tested a week later with the unemulsified fat preparation.

Results. The results revealed no statistical difference between normal dogs and dogs with bile exclusion tested with I^{131} labeled unemulsified fat (Fig. 1). Likewise, there was no significant difference between normal and bile duct occluded dogs tested with I^{131} labeled emulsified fat (Fig. 2). There was, however, a striking difference in radioisotope blood level in normal and in pancreatectomized dogs tested with I^{131} labeled emulsified fat (Fig. 2).

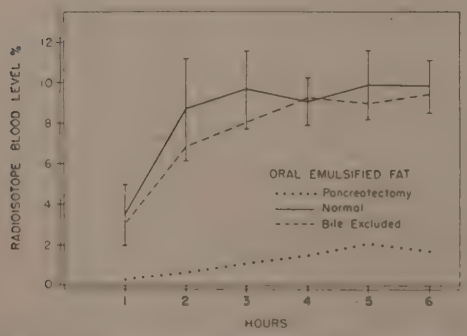

FIG. 2. Graph showing comparison in mean curve of blood isotope percent in 14 normal, 14 bile duct occluded, and 10 pancreatectomized dogs, all tested with an I^{131} labeled emulsified fat test meal.

Previous studies, using different technics have indicated that bile is quantitatively concerned in the digestion and absorption of fat (3-5) and that exclusion of bile reportedly results in decreased fat absorption(6). Further, previous studies have indicated that exclusion of pancreatic secretion produces a more marked alteration of fat digestion and absorption. Several investigators have used an iodized fat in studying fat digestion(7-10). Earlier studies(11-13) showed that although an iodized fat is unnatural, its digestion, absorption and metabolic fate closely followed that of neutral fat after oral administration. The glycerol trioleate used in the present study is a long chain, predominantly saturated fat. The peanut oil is a mixture consisting of mainly unsaturated fatty acids of high iodine index. From a chemical standpoint the bond between the I^{131} and the fat molecule theoretically is a firm one. Although the exact nature of fat digestion and absorption is not known, one theory holds that when a fat, as used in this study, enters the normal gut, hydrolysis of the fat molecule occurs, yielding glycerol and fatty acids, to which the I^{131} remains bound. Glycerol and fatty acids supposedly then re-unite in the wall of the gut to form again a fat molecule before entrance into the lacteals. Studies in this laboratory, to be reported elsewhere, show that undigested portion of administered fat passes in the stool, carrying with it the equivalent proportion of bound I^{131}. Furthermore, pancreatectomy had no influence on rate of absorption of ionic I^{131}, but has a profound influence on the rate of entrance of I^{131} into blood when it is chemically bound to the fat molecule.

The present study indicates that addition of an emulsifier produced little or no effect on fat digestion and absorption in the normal animal. Exclusion of bile made little difference in rate and in total amount of radioactive labeled fat entering the blood during the 6-hour period of the test, whether the fat was emulsified or unemulsified. On the other hand, exclusion of pancreatic juice caused a marked depression in the amount of radioactive labeled emulsified fat which entered the blood during the test period.

Summary. Using a fat test meal containing a radioactive labeled fat, a study has been carried out in dogs to assess the rate and total amount of radioactive labeled fat entering the blood in normal dogs and in dogs with exclusion of bile and pancreatic juice during a 6-hour period after administration of the meal. These studies indicate that bile plays a minor role and pancreatic juice a major role in fat digestion and absorption. This study suggests the use of a similar test for clinical use in evaluating pancreatic function in suspected pancreatic disease.

1. Shingleton, W. W., Wells, M. H., Baylin, G. J., Ruffin, J. M., and Sanders, A., *Surgery,* 1955, v38, 134.

2. Markowitz, J., Archibald, J., and Downie, H. G., *Experimental Surgery,* 3rd edition, Baltimore, 1954, Pp. 355-356.

3. Muller, Fredriek, *Z. f. klin. Med.,* 1887, v12, 45.

4. Munk, Immanuel, *Virchows Arch. f. path. Anat.,* 1890, v122, 302.

5. Hedon, E., and Ville, J., *Arch. de physiol. norm. et path. S. s.,* 1897, v9, 606.

6. Coffey, R. J., Mann, F. C., and Bollman, J. L., *Am. J. Dig. Dis.,* 1940, v7, 143.

7. Groen, J., *Am. J. Med.,* 1948, v4, 814.

8. Van Creveld, S., *Nederl. tijdschr. v. geneesk.,* 1927, v71, 2015; 1931, v75, 2795.

9. Tremolieres, F., and Cheramy, P., *Concours medical,* 1932, v59, 3241.

10. Silverman, F. N., and Shirkey, H. C., *Pediatric,* 1955, v15, 143.

11. Pellegrini, H., *Atti. d. r. Accad. d. Fisiocrit. in Siena,* 1947, v13, 290.

12. Artom, C., and Peretti, G., *Arch. internat. de physiol.,* 1933, v36, 101.

13. Von Bodo, R., and Schaeffer, L., *Arch. f. exp. Path. u. Pharmakol.,* 1927, v124, 326.

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Experimental Hydro-uteri (Hydrometra) in Rodents and Some Factors Determining Their Formation.* (22148)

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In mice of the English Silver Strain, and occasionally in other strains there occurs a condition of imperforate vagina which leads to a marked distention of uteri with accumulation of fluid up to 15 ml or more per mouse. Yet normal function of the uterus is preserved, since such animals become pregnant if the membrane occluding the vagina is removed(1). Existence of huge hydro-uteri in the presence of imperforate vaginas suggested that simple ligation of the cervix in rodents might likewise induce formation of hydro-uteri. This new technic not only provides a large uterine receptacle for tumor transplants but also permits the collection of large amounts of uterine secretions in small animals.

This paper reports studies on production of hydro-uteri in mice, rats and hamsters and on hormonal and other factors which play a role in development of experimental hydro-uterus.

Methods. The cervix of the uterus was ligated under nembutal anesthesia through a supra-pubic incision in 456 mice, in a smaller number of rats (Wistar) and Syrian hamsters. The variables for this study of effects on development of hydro-uteri were strain of mouse, endocrine status and time elapsing between uterine cervix ligation and autopsy. The strains of mice and types of animals used are shown in Tables I and II. Eighty-four mature white Swiss mice, 40 DBA/1 and 40 C57BL/10J mice were castrated and their cervices ligated (Table I). Forty-seven immature intact white Swiss mice underwent

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TABLE I. Hydro-uterus Formation in Immature and in Castrated Animals.

	No. of animals	Strain	Age, mo	Duration of ligation (D)	% hydro-uteri		
Intact immature animals	47	Wh. Sw. Tufts	½–1	13–28	21		
Castrated mature animals	44	Wh. Sw. Tufts	3–8	15–60	40		
					Castrate control	Testosterone (.6 mg)	Estradiol (12 γ)
Castrated mature animals	40 10*	Wh. Sw. RJB stock	2½	21	0	0	30
	40 10*	DBA/1 RJB stock	2½	21	0	0	20
	40 10*	C57BL/10J	2½	21	0	0	20

* In each category.

TABLE II. Hydro-uterus Formation in Intact Mature Animals.

	No. of animals	Strain	Age (mo)	Duration of ligation (D)	% hydro-uteri
Mice	163	Wh. Sw. Tufts	3–12	21–180	33–(66)
	51	BBF1	3– 7	21–190	59–(90)
	12	C57BR/a	6	24	33
	35	Wh. Sw. RBJ stock	2½	22	20
	42	C57BL/H-2d	2½	42	29
	26	DBA/1 RBJ stock	3	30	35
Rats	10	Wistar rats	4	22	30
Hamsters	1	Golden Nugget	2	35	Positive

cervical ligation. One to 2 weeks later, castrates received injections subcutaneously as follows: *controls,* 0.5 ml sesame oil; *estrogen treated animals,* 1 γ estradiol daily; *testosterone treated animals,* 0.1 mg testosterone propionate every other day for 12 days. Twelve days later these animals were sacrificed. The effect of length of time after ligation of the cervix was studied in mature intact animals by performing exploratory laparotomies at varying intervals (up to 12 months) after cervical ligation. At the end of the experiments the animals were sacrificed and histological studies made of the uteri. The fluid which accumulated in the uteri was harvested and analyzed† for the following constituents by the indicated methods: for proteins(2), carbohydrates(3,4), hexosamine(5) and hexuronic acid content(6). Bacteriological and cytological studies were also carried out. For purposes of comparison fluid from the hydro-uterus of a white Swiss mouse with imperforate vagina, ascitic fluid from Ehrlich's ascites tumor growing in white Swiss mice and from a foreign body ascites provoked by intraperitoneal injection of Walker (rat) Sarcoma cells into the abdomen of mice was similarly analyzed.

Results. The data obtained are summarized in Tables I, II and III.

Hydro-uterus formation in intact mature animals. In Table II approximately one-third of all animals which had undergone ligation of their uterine cervix developed distended uterine horns containing at least 0.5 ml of fluid 21 days after ligation. It is not clear why the remaining two-thirds of these

† We are indebted to Drs. W. H. Fishman and J. Nisselbaum for these analyses.

TABLE III. Chemical Composition of Various Hydro-Uterine and Ascitic Fluids.

Fluid pools studied	Protein content(2) (mg/ml) Ppt'd by $HClO_4$	Protein content(2) (mg/ml) Ppt'd by phosphotungstate	Carbohydrates in the phosphotungstate precipitate: Carbohydrate content (3,4) (mg/ml) expressed as half mannose, half galactose	Carbohydrates in the phosphotungstate precipitate: Hexosamine(5) content (mg/ml)	Carbohydrates in the phosphotungstate precipitate: Hexuronic acid(6) content (mg/ml)
HF* from WSM No. 4 7 mo after uterine ligation	trace	12.2	3.8	2.5	.15–.25
HF from WSM No. 4 11 mo after uterine ligation	trace	5.2	1.6	.77	.4 –.5
HF from mouse with imperforate vagina	30.0	33.5	2.8	3.6	
	(approx. values; total is 63.5 mg/ml)				
AF from Ehrlich Ascites tumor	52.2	2.9	.24	1.2	0
AF from ♂ WSM with Walker tumor abdom.	34.2	1.5	.13	.6	.17

* HF = Hydrouterine fluid; WSM = White Swiss mouse; AF = Ascitic fluid.

animals fail to accumulate fluid in distended horns and show reasonably normal uterine horns in spite of cervical ligation. The possibility that this is merely a result of chance variations in the surgical technic can not be ruled out. Simultaneous ligation of the 2 oviducts in 136 mice and 7 rats did not increase significantly the frequency of hydro-uteri. The most important single factor favoring development of hydro-uteri is time, since in 2 groups of 12 animals, studied for 6 months, from 66 to 90% showed large hydro-uteri (Fig. 1). Fluid continued to accumulate rapidly in spite of periodic emptying of the hydro-uterus by aspiration of the fluid by needle puncture.

The tendency to formation of hydro-uterus following cervical ligation is not clearly determined by genetic factors since non-inbred white Swiss mice (Tufts), inbred white Swiss mice (Jackson, C57Br/a, C57BL/H-2d and DBA/1 mice all behaved essentially in the same way. It appears, however, that the BBFl mice tend to have a greater incidence of hydro-uteri under otherwise similar experimental conditions. Ligation of the cervices of rats produced the same results as in mice. A hydro-uterus was also obtained in a hamster.

Chemical analysis of various hydro-uterine fluids yielded results shown in Table III. It appears that the protein content (precipitated by $HClO_4$) of hydrouterine fluid in experimental hydro-uteri is considerably lower than that in ascites fluids and that the mucopolysaccharide content as reflected by the phosphotungstate precipitable proteins and carbohydrates of the phosphotungstate precipitate is higher. In fluid collecting in the uterus with imperforate vagina the protein content is higher than in ascites fluid. Bacteriological studies showed that all fluids studied were sterile.

Studies on the effects of hormonal factors are shown in Table II. Within the same period of time immature white Swiss mice showed the same incidence of hydro-uteri as mature intact animals. Castrated white Swiss mice observed for the same length of time (21 days) had no hydro-uteri, but after 60 days formed as many or more hydro-uteri as did intact controls (Tables I and II).

In other strains of mice, namely in inbred white Swiss mice, DBA/1 and C57BL/10J mice, castration prevented formation of hydro-uteri but they formed again when estradiol was given. However, it was found that if the castrated, untreated animals were again examined 2 or 3 months after ligation, many had formed hydro-uteri. Castration merely slows development of hydro-uteri but does not prevent it. Testosterone did not prevent or alter the retarding effect of castration, but

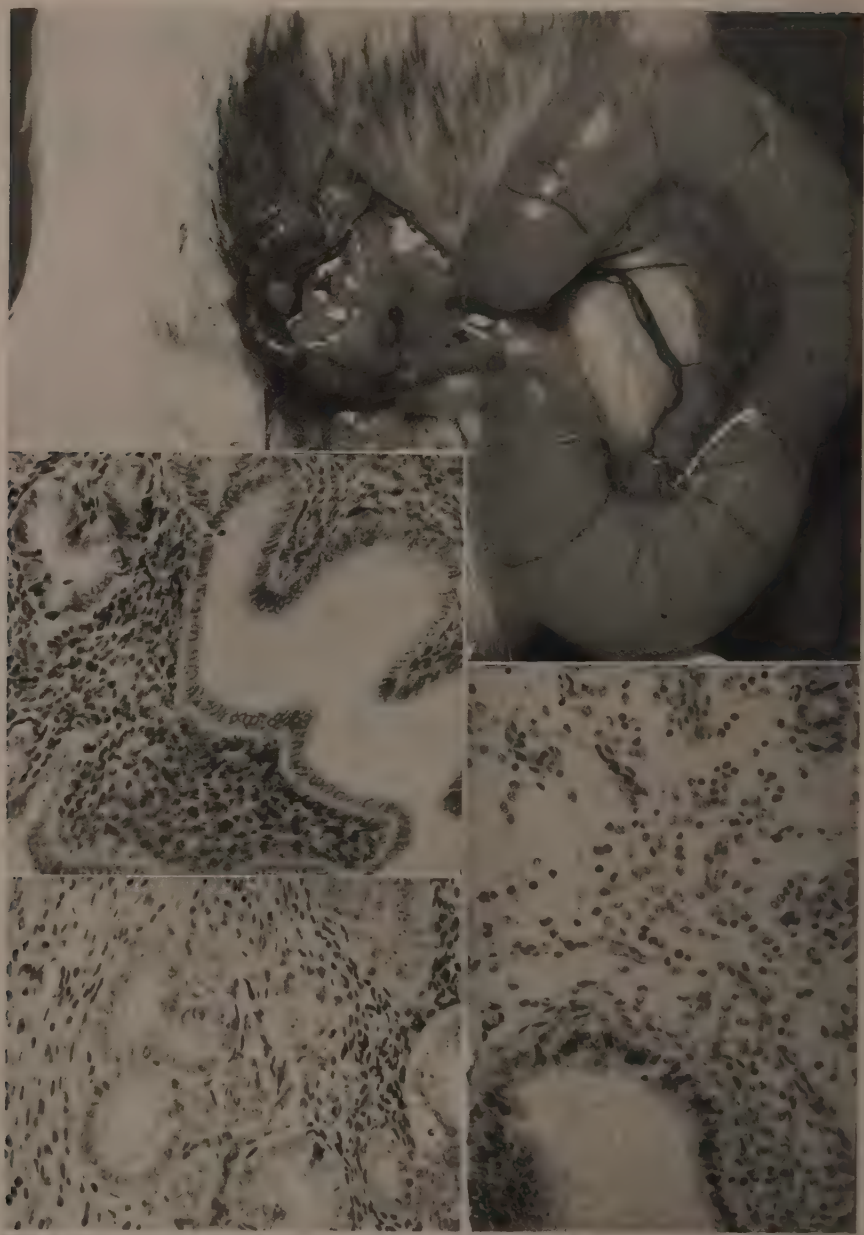

FIG. 1. Top: Appearance of unilateral hydrouterus in a white Swiss mouse, 9 mo of age, 8 mo after ligation of left uterine horn, near cervix. Compare size of hydrouterus with that of non-ligated horn. Left center: Microphotograph of hydrouterus in normal control animal, 21 days after ligation. Left bottom: Microphotograph of hydrouterus in estrogen treated castrate, 21 days after ligation (same magnification). Right bottom: Microphotograph of hydrouterus in testosterone treated animal, 21 days after ligation (same magnification).

estradiol given to castrates restored the rate of formation of the hydro-uterus.

Histological examination of uteri after cervical ligation showed that the wall of the uterus, the endometrium and the endometrial glands react normally to stimulation by estrogens (Fig. 1). Testosterone produced atrophy of the glandular elements of the uterus and edema of the uterine wall with rarification of its cellular elements (Fig. 1). In all instances studied histologically there was complete absence of any inflammatory reaction in the uterine wall.

Discussion. Ligation of the cervix of mice, rats and hamsters leads to accumulation of a fluid in the uterus which can be harvested by uterine puncture and which keeps accumulating after each consecutive tap. As much as 100 ml of hydro-uterine fluid has been obtained from a single mouse during a year's time . The hydro-uteri provide an ideal site for tumor growth(7).

Endocrine imbalance produced by castration retards formation of hydro-uteri but does not prevent it, and administration of estrogen restores the rate of accumulation of intra-uterine fluid to normal. In immature mice the incidence of formation of hydro-uteri is reduced to about one-half of that seen in normal mature animals of the same strain.

Characteristics of hydrouterine fluid are different from those found in ascitic fluid, since the protein content is low and carbohydrate is high. This feature of hydrouterine fluid is compatible with the characteristics one would expect from a collection of endometrial secretions. If the collecting fluid is allowed to accumulate for several months, the relative proportions of its main components do not change but their concentrations are lowered, indicating some dilution taking place with time. It is believed that the experimental production of hydro-uteri in rodents constitutes a new method for the collection of endometrial secretions.

1. Gowen, J. W., and Heidenthal, G., *J. Exp. Zool.*, 1942, v89, 433.

2. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, 1940, v135, 727.

3. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.

4. Sorenson, M., and Haugaard, G., *Biochem. Z.*, 1933, v206, 247.

5. Rimington, C., *Biochem. J.*, 1940, v34, 931.

6. Dische, Z., *J. Biol. Chem.*, 1947, v167, 189.

7. Homburger, F., Tregier, A., and Grossman, M. S., *Cancer Research*, in press.

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Estimation of Bromide on Fingertip Blood in Bromide Intoxication. (22149)

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On several occasions it has been necessary to follow bromide levels of serum of small infants after bromide administration either therapeutically or accidentally. The method usually employed (bromide as gold bromide) requires amounts of serum greater than is conveniently obtainable from capillary blood. This paper describes a procedure practical for fingertip blood. The gold bromide procedure is used except that readings are taken in ultraviolet where a higher absorption peak exists for gold bromide.

Method. Reagents and materials. The contents of a one g. vial of gold chloride ($AuCl_3 \cdot HCl \cdot 3H_2O$) 0.25%, is washed into distilled water and made up to 400 ml. Trichloracetic Acid: 20 g is made up to 100 ml with distilled water. Carbon Tetrachloride: Analytical reagent grade. Bromide Standard (100 mg/100 ml): 641 mg of sodium bromide (dried overnight in vacuum desiccator) is made up to 500 ml with distilled water. Screw Cap Tubes: Kimble #45066, 16 x 125 mm, fitted with screw caps, teflon lined, are rinsed with dilute nitric acid, distilled water, and dried.

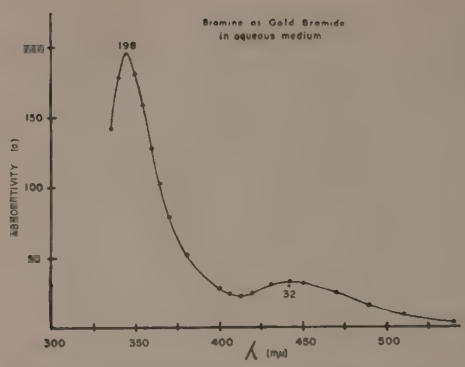

FIG. 1. Absorption spectrum of gold bromide in trichloracetic acid, under conditions of the procedure described. Beckman spectrophotometer.

Procedure: Add 0.2 ml distilled water to screw cap tube. Wash in 0.05 ml of unknown serum. Add 1.3 ml of 20% trichloracetic acid, mix. Follow with 1 ml carbon tetrachloride. Cap the tube and shake vigorously by hand or shaking machine for a few minutes and centrifuge 5 minutes at 2000 r.p.m. in a #1 International centrifuge or its equivalent. Transfer 1 ml aliquot from top layer to the quartz cuvet (1.5 ml capacity, 1 cm light path)*. Add 0.2 ml of 0.25% gold chloride, mix and read on Beckman Spectrophotometer at 350, or 440 mμ if optical density is greater than 0.8. For the standard, use 0.05 ml of standard instead of serum. For the blank, use 0.05 ml distilled water instead of serum. Calculations: Read bromide concentration off standard curve. For clinical purposes the formula below will yield sufficiently accurate results:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 100 = \text{mg/100 ml bromide in unknown serum}$$

Results. Fig. 1 illustrates the absorption curve of gold bromide which exhibits a peak at 350 mμ and at 440 mμ. The latter is the peak customarily employed(1,2). By reading in the ultraviolet an increase in sensitivity by a factor of approximately 6 is obtained.

The concentration of gold affects the shape of the absorption spectrum. At higher gold concentrations the peak shifts toward the higher wave lengths. If the gold concentration is doubled the ultraviolet peak moves to 355 mμ. The peak at 440 mμ is not affected by changes in gold concentration.

Location of the lines in Fig. 2 were obtained by applying the method of least squares(3) to 6 determinations, at each level shown, to find the line of best fit. It is to be noted that both at 350 mμ and at 440 mμ the lines do not go through the origin. Thus the method may not be used for normal serum where concentrations are of the order of 0.1 mg bromide per 100 ml(4).

A line may be drawn through the origin passing near the points. Thus the Beer-Lambert law may be applied, approximately, when dealing with bromide intoxication, since the toxic level is above 80 mg bromide/100 ml serum(5).

Gold chloride and bromide complex is extractable into higher alcohols and ketones at strongly acid pH but not into other solvents such as chloroform, benzene or heptane. Fig. 3 shows absorption spectrum of gold bromide

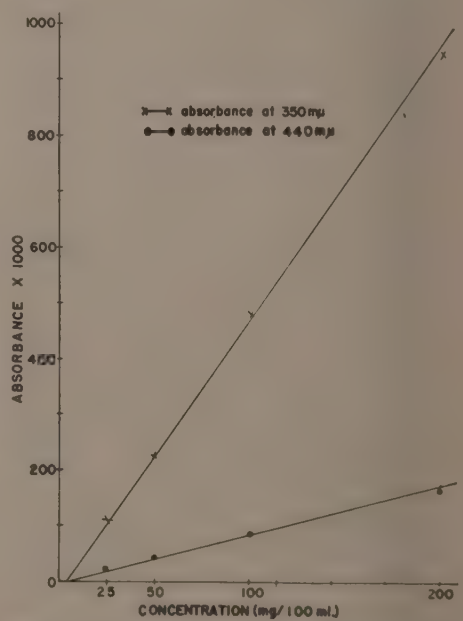

FIG. 2. Calibration curve plotting concentration vs. absorbance. Each point is the mean of 6 determinations. Position of line calculated by method of least squares. Concentration refers to concentration of the serum.

* Pyrocell Mfg. Co., N. Y. City.

extracted into octyl alcohol from a 10% sulfuric acid solution. Attempts to extract the bromide quantitatively, without first precipitating proteins, were not successful, and this method, then, had no advantage over the aqueous method for serum or plasma analysis. This procedure would find application when small amounts of bromide are present in large aqueous volumes, the volume of octyl alcohol layer is then limited to a small volume.

Filtrates with trichloracetic acid tend to be cloudy, occasionally. This is true especially when serum is first diluted with water before adding trichloracetic acid. This is avoided by adding carbon tetrachloride when precipitating the proteins as described in the procedure.

Table I shows recoveries of added bromide to a pooled sample of serum at different levels. High recoveries for the lower quantities are probably due to substances in the centrifugate other than bromine reacting with the gold chloride. In the high range, disappearance of appreciable amounts of gold lowers recovery values. In these high ranges it is suggested that smaller amounts of serum (0.02 ml) be used. The results at 350 mμ compare with those at 440 mμ, indicating that readings at 350 mμ permit estimation of smaller quantities of bromide without loss in precision. When absorbance at 350 mμ is higher than 0.800, then one may read at the less sensitive peak, 440 mμ. This permits a wide range of concentrations to be read without the need of repeating the analysis with smaller quantities.

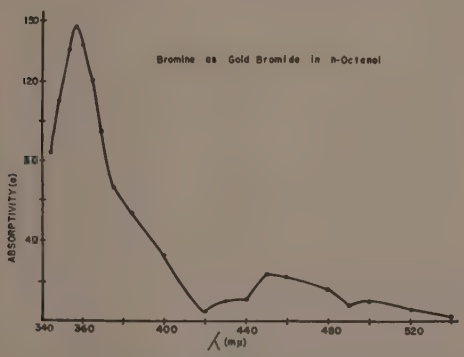

FIG. 3. Absorption spectrum of gold bromide extracted from 10% sulfuric acid into n-octanol. Beckman spectrophotometer.

TABLE I. Recovery of Bromide Added to Serum.

Wave length (mμ)	μg added	μg found*	% recovery
350	12.5	13.1 ± 1.8†	104
"	25	26.6 ± 2.3	104
"	50	51.2 ± 4.4	102
"	100	98.5 ± 6.5	98.5
440	25	26.1 ± 3.4	104
"	50	51.1 ± 4.9	102
"	100	97.0 ± 5.8	97

* Six determinations at each level.
† Mean ± stand. dev.

Summary. A procedure is described for bromide analysis in serum of patients with bromide intoxication which uses the ultraviolet absorption peak for gold bromide to increase sensitivity. This permits the analysis of serum or plasma, from fingertip blood, for bromide analysis.

1. Barbour, R. F., Pilkington, F., and Sargent, W., *Brit. Med. J.*, 1936, v2, 957.

2. Paul, W. D., Knouse, R. W., and Routh, J. I., *J. Am. Pharm. Assn.*, 1952, v41, 205.

3. Thomas, G. B., Jr., *Calculus and Analytic Geometry*, Addison-Wesley Press, Cambridge, Mass., 1951, pp. 538-540.

4. Serbescu, P., and Buttu, G. A., *Bull. Acad. Med.*, 1934, v111, 232.

5. Perkins, H. A., *Arch. Int. Med.*, 1950, v85, 783.

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Histamine Sensitivity of Adrenalectomized and Pertussis-Treated Mice. (22150)

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It has been established that mice, which are normally resistant to histamine, become highly sensitive after being injected with *Hemophilus pertussis* vaccine (see refs. in 1-10). The mechanism by which this vaccine increases the sensitivity to histamine by 25 or 100 times remains unknown, although several authors have suggested that diminished function of adrenal cortex and medulla may be involved since adrenalectomized mice are known to be sensitive to histamine (usually injected intraperitoneally)(10-14), and sensitivity is partially or completely lost after treatment with epinephrine and cortisone (5,14). It seemed pertinent to make a quantitative study to determine the degree of histamine sensitivity which develops in mice following treatment with pertussis-vaccine as compared directly with the sensitivity following adrenalectomy. The results of this study revealed that the sensitivity to histamine induced with pertussis vaccine could scarcely be related to altered function of the adrenal gland since adrenalectomized mice of the strain used were no more sensitive to intravenous injections of histamine than normal mice, whereas pertussis treatment of intact mice enhanced sensitivity to histamine 33 times.

Methods. The general plan of the experiment was to determine concurrently the LD_{50} of histamine diphosphate in normal, adrenalectomized and pertussis-treated mice in order to make a direct comparison of histamine sensitivity in the 3 types of animals. Female albino mice of the Swiss-Webster strain (Taconic) weighing between 15 and 27 g were obtained from a local source, and maintained before and after operation or vaccine injection on a diet of Purina Laboratory Chow. Bilateral adrenalectomies were conducted under light ether anesthesia and operated mice were provided with 0.9% saline until injected with histamine the following day. All other mice were supplied with tap water *ad lib.* *Hemophilus pertussis* vaccine supplied by the Massachusetts State Health Department contained 20 billion Phase I organisms per ml and Merthiolate in concentration of 1:10,000. This was diluted with saline so that each 0.5 ml contained 8.75 billion organisms, this volume being injected intraperitoneally in mice 4 days prior to histamine injection. Mice in control groups were intact and untreated at the time of histamine injection. Histamine diphosphate solutions were prepared in normal saline, then several dilutions in saline were made to obtain concentrations which permitted injection of various doses (Table I) in constant volume of 0.01 ml per gram of mouse. Histamine solutions were refrigerated and used within 48 hours of preparation. Injections of histamine were made into the tail vein during a period of 10 seconds, 4 graded doses being injected into normal, adrenalectomized and pertussis-treated mice on each day of experimentation. Each dose of histamine was injected into 20 mice, with exceptions as indicated in Table I.

Completeness of adrenalectomy was checked by providing a few mice from each operated group with 0.9% saline for 24 hr and tap water thereafter, incidence of mortality being recorded during 14 days. Some of these mice were fed the Purina Chow, others were fed oatmeal which has a lower sodium content. Histamine injections were made in adrenalectomized mice 24 hr after operation in order to minimize the possibility of growth of adrenal rests. Incidence of mortality was recorded as the number of mice dead per total number injected for a given dosage level after 24 hours. LD_{50} values were computed according to the method of Litchfield and Wilcoxon(15).

TABLE I. Acute Toxicity of Histamine Diphosphate in Normal, Adrenalectomized and Pertussis-Treated Mice as a Measure of Histamine Sensitivity.

Treatment of mice	Histamine diphosphate, mg/kg, I.V.	Mortality Ratio	Mortality %	LD_{50}, mg/kg, I.V. (fiducial limits) 95%
Normal (controls)	300	4/20	20	333
	350	9/20	45	(309-367)
	400	16/20	80	
	550	16/16	100	
Adrenalectomized	150	0/ 6	0	315
	250	1/20	5	(284-350)
	300	12/20	60	
	350	13/20	65	
	400	15/20	75	
Pertussis-treated	6	2/20	10	10
	8	8/20	40	(8.8-12.3)
	10	8/20	40	
	14	14/20	70	

Results. Data presented in Table I reveal that pertussis-treated mice were 33 times more sensitive than normal mice to histamine injected intravenously, as indicated by LD_{50} values of 10 and 333 mg/kg, respectively. However, the adrenalectomized mice were no more sensitive than normal mice to histamine injected intravenously, since the LD_{50} values of 315 and 333 mg/kg, respectively, are almost identical.

Failure of adrenalectomized mice to exhibit sensitivity to histamine injected intravenously could not be due to incomplete adrenalectomy or growth of adrenal rests. A majority of the mice were considered to be completely adrenalectomized because: a. 63% of 71 adrenalectomized mice provided with Purina Chow and tap water died in 14 days; 94% of 16 adrenalectomized mice died in 14 days when provided with tap water and oatmeal, the latter having a lower sodium content than Purina Chow. b. Histamine was purposely injected 24 hr after adrenalectomy, thus eliminating the possibility of significant growth of adrenal rests. c. Furthermore, an experiment was made which confirmed the reports that adrenalectomized mice become sensitive to histamine injected intraperitoneally(10-14). Intraperitoneal injection of graded doses of histamine in 40 adrenalectomized mice revealed that they were 3 to 7 times as sensitive as normal mice.

Discussion. The evidence presented clearly indicates that pertussis-treatment greatly increased sensitivity to histamine in the strain of mice used. It is clear that this strain of mice did not become sensitive to histamine injected intravenously after complete adrenalectomy. The failure of these adrenalectomized mice to exhibit sensitivity to histamine is not a unique finding since a CF-1 strain of mice was shown not to be sensitive after either injections of pertussis vaccine(10) or adrenalectomy(13). The important point indicated by the data herein presented is that the mice became sensitive to histamine after injection of pertussis vaccine, but not after adrenalectomy. This experiment therefore provides definite evidence that sensitivity to histamine which developed after treatment with pertussis vaccine cannot be ascribed to diminished function of the adrenal cortex and/or medulla, or to diminished action of hormones from the adrenal gland.

Although several authors have suggested that histamine sensitivity induced by pertussis vaccine is due to an injurious action of the vaccine on the adrenal glands, there has been no direct evidence presented to support the belief. If pertussis vaccine diminished the function of the adrenal medulla and cortex in mice one would expect the animals to become susceptible to various forms of stress. However, Kind(4) states that pertussis-treated mice did not become sensitive to injections of methacholine. Furthermore, the susceptibility to severe hypoxia(6) was accompanied by liver glycogen values which were not in-

dicative of abnormal adrenal cortical function. No evidence was presented to implicate abnormal adrenal function as the cause of hypoglycemia which developed in pertussis-vaccine treated mice(2). Other evidence exists which suggests the possibility that pertussis vaccine causes dysfunction of the adrenal glands. The sensitivity to histamine (injected I.P.) in pertussis-treated and adrenalectomized mice is partially or completely restored by proper treatment with cortisone (5) or a combination of cortisone and epinephrine(14). Nevertheless, the effects of these substances may be pharmacological in nature rather than specific physiological effects of replacement therapy after adrenal inactivation.

The authors are not aware of published data which prove that pertussis-vaccine alters the adrenal cortex as indicated by cholesterol content, ascorbic acid depletion, or other tests of adrenal function.

Summary. In a Swiss-Webster strain of mice, pertussis vaccine treatment increased the sensitivity to intravenously injected histamine 33 times, whereas surgical adrenalectomy had no significant effect on sensitivity to histamine. This finding, in addition to lack of direct evidence to the contrary, warrants the conclusion that *Hemophilus pertussis* vaccine enhances sensitivity of mice to histamine through some mechanism other than impairment of adrenal function.

1. Parfentjev, I. A., and Goodline, Mary A., *J. Pharmacol. Exp. Therap.*, 1948, v92, 411.
2. Parfentjev, I. A., and Schleyer, W. L., *Arch. Biochem.*, 1949, v20, 341.
3. Malkiel, Saul, and Hargis, Betty J., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 109.
4. Kind, L. S., *J. Immunol.*, 1953, v70, 411.
5. ———, *J. Allergy*, 1953, v24, 52.
6. Kind, L. S., and Gadsden, R. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 373.
7. Pittman, M., and Germuth, F., *ibid.*, 1954, v87, 425.
8. Parfentjev, I. A., *ibid.*, 1955, v89, 297.
9. Maitland, H. G., Kohn, R., and MacDonald, A. D., *J. Hygiene*, 1955, v53, 196.
10. Munoz, J., and Schuchardt, L. F., *J. Allergy*, 1953, v24, 330.
11. Halpern, B. N., and Wood, D. R., *Brit. J. Pharmacol.*, 1950, v5, 510.
12. Saunders, F. J., *Endocrinology*, 1951, v49, 185.
13. Munoz, J., and Schuchardt, L. F., *J. Allergy*, 1953, v25, 125.
14. Halpern, B. N., Benacerraf, B., and Briot, M., *Brit. J. Pharmacol.*, 1952, v7, 287.
15. Litchfield, J. T., Jr., and Wilcoxon, F. W., *J. Pharmacol. Exp. Therap.*, 1949, v96, 99.

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Diet and TEPA Therapy in Sarcoma-Bearing Rats.* (22151)

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Data have been presented to demonstrate that N, N′, N′′ triethylenephosphoramide (TEPA) reduced food efficiency and growth of normal as well as tumor tissue in tumor-bearing rats(1). Given an optimum dosage and a susceptible tumor, however, reduction in growth of the tumor was greater than reduction in weight of normal tissues(2). These data also demonstrated that a high protein diet, or a low casein diet supplemented with methionine, reduced the depleting effect of TEPA on normal tissue without altering the deleterious effect on the tumor(1,2). Other experiments have demonstrated that supplementing a low casein diet with a mixture of methionine and guanidoacetic acid or methionine and glycine resulted in increased protection of normal tissues to the depleting effects of the tumor(3). Further work has been done to determine the effect of these mixed supplementations upon chemotherapy of TEPA in tumor-bearing rats.

* Some of these results were read before the Am. Chem. Soc. Meeting in New York, Sept. 1954. These studies were supported by an Institutional Grant from the American Cancer Society.

TABLE I. Effect of Various Supplementations in Diet Containing 12% Casein upon TEPA Therapy (1 mg/Day/kg Body Wt). Food efficiencies were calculated as grams gain in body wt (tumor wt subtracted) per 100 g of food intake.

Diet	Food efficiency	Tumor wt, g	Gain in body wt/tumor
	Avg data using 10 animals/group		
Casein 12% (no TEPA)	8.6	36.7	1.41 ± .26*
Casein 12% + TEPA	4.1	10.9	1.97 ± .39
+ glycine (Gl) + TEPA	4.4	9.7	2.29 ± .24
+ alanine (Al) + TEPA	4.2	10.7	2.02 ± .44
+ NH_4 citrate (NH_4) + TEPA	4.2	8.4	1.50 ± .86
+ methionine (M) + TEPA	5.4	8.4	3.24 ± .67
+ M + NH_4 + TEPA	4.0	8.2	2.76 ± .80
+ M + Al + TEPA	5.7	13.3	3.00 ± .50
+ M + Gl + TEPA	5.4	7.2	3.61 ± .30
+ M + guanidoacetic acid + TEPA	8.1	7.2	5.64 ± 1.22
Casein 12% + TEPA	4.2	6.9	1.95 ± .67
+ M + TEPA	5.9	5.4	3.10 ± .96
+ M + NH_4 + TEPA	6.2	7.1	3.37 ± .95
+ M + Gl + TEPA	6.0	7.1	3.15 ± .98
+ M + casein 1.5% + TEPA	6.1	7.5	3.04 ± .78
+ M + guanidoacetic acid + TEPA	9.6	4.6	6.57 ± 1.12
	Avg data using 58 animals/group		
Casein 12% + TEPA	4.0	7.7	2.47 ± .59
+ M + TEPA	7.9	7.3	4.37 ± .74
+ M + guanidoacetic acid + TEPA	10.31	5.5	7.62 ± 1.00

* Stand. error.

Methods. Transplanting the sarcoma in the rat and measuring growth of the tumor have been described previously(1). The tumor developed most rapidly in rats fed a semi-synthetic diet containing 12% casein, a diet that has been reported in detail(1). The effect of various types of supplementation of the diet on growth of the body and the sarcoma in the presence of TEPA is reported in this paper. These supplements were 0.67% DL methionine and 0.67% DL methionine plus 0.67% guanidoacetic acid. These percentages and mixtures had been demonstrated to be optimum for growth of normal tissue in tumor-bearing rats(3,4) and for growth in weanling rats(5) fed 12% casein diet. Glycine (1.28%), alanine (1.53%), ammonium citrate (1.95%) and casein (1.5%) which contain nitrogen in amounts equivalent to that found in guanidoacetic acid (0.67%) were studied also as single supplements and combined with methionine. Food efficiencies were calculated as grams gained in body weight (tumor weight subtracted) per 100 g of food intake. TEPA was injected intraperitoneally as 1 mg/day/kg of body weight after tumor had reached approximately one g in weight and was well established. Ten rats were used to determine the effect of each supplement on TEPA therapy.

Results. Data recorded in Table I illustrate food efficiencies, tumor weights and Δ body weight/tumor ratios approximately 3 weeks after transplantation of the sarcoma. The rate of increase in body with respect to the tumor is taken as one measure of the effect of the tumor on the body. At the end of 3 weeks the tumor had reached lethal proportions (36.7 g) in rats not given TEPA. The Δ body/tumor ratio was low (1.41) indicating a depleted body. The TEPA therapy (1 mg/day/kg body weight) was started in other groups of rats after the tumor had reached about one g in size and was well established. This therapy reduced growth of tumor to 10.9 g in rats fed 12% casein diet over this 3-week period but also reduced food efficiency and growth of the body of the animal. Supplementing the 12% casein diet with glycine, alanine or ammonium citrate did not alter results significantly. Supplementing with methionine, however, did increase food efficiency and rate of growth of the body with respect to the tumor in the

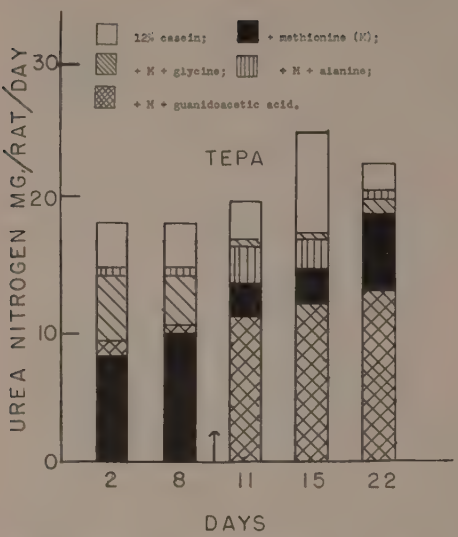

FIG. 1. Effect of different diets on excretion of urea nitrogen in sarcoma-bearing rats. All values start from base. TEPA therapy was instituted at time indicated by arrow.

presence of TEPA. Supplementing with methionine plus guanidoacetic acid increased food efficiency and rate of growth of the body still further. Adding an equivalent amount of nitrogen to guanidoacetic acid in the form of glycine, alanine, ammonium citrate or casein with the methionine did not have the marked effect noted with guanidoacetic acid, results which suggest a specific role for this acid.

The possibility that supplementation with a mixture of methionine and guanidoacetic acid could reduce catabolic activity of tissue proteins was suggested. The white bars in Fig. 1 record excretion of urea nitrogen in tumor-bearing rats fed 12% casein, data that illustrate the high excretion of urea nitrogen associated with a nutritionally malignant tumor. The black bars illustrate reduction in excretion of urea nitrogen in rats fed the casein diet supplemented with methionine. The bars with slanted lines record excretion in the presence of methionine plus glycine while bars with vertical lines record excretion of urea nitrogen in the presence of methionine plus alanine. Adding glycine or alanine increased excretion of urea, probably reflecting the catabolism of these amino acids. The bars with crossed lines illustrate excretion of urea nitrogen in tumor-bearing animals fed the casein diet supplemented with both methionine and guanidoacetic acid. Thus methionine plus guanidoacetic acid reduced excretion of urea nitrogen markedly during TEPA therapy, a reduction that may be correlated with better gain in body weight. These results emphasize the importance of combining nutritional therapy with chemotherapy.

Summary. Data have been presented to demonstrate that TEPA reduced food efficiency and growth of body weight as well as tumor weight in sarcoma-bearing rats fed a 12% casein diet. Supplementing with methionine and guanidoacetic acid increased food efficiency and growth of the body without increasing the growth of the tumor. This increase in growth of the body is correlated with reduction in excretion of urea.

1. Allison, J. B., Wannemacher, Jr. R. W., Hilf, R., Migliarese, J. F., and Crossley, M. L., *J. Nutrition,* 1954, v54, 593.

2. Crossley, M. L., Allison. J. B., Parker, R. P., Kuh, E., and Seeger, D. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 438.

3. Allison, J. B., Hilf, R., and Wannemacher, Jr. R. W., *Fed. Proc.,* 1955, v14, 426.

4. Hilf, R., Rutgers University Ph.D. Thesis, 1955.

5. Baron, H., and Allison, J. B., *Fed. Proc.,* 1954, v13, 450.

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